Studies on the behaviour and associated neural and muscular organization of cercariae of Transversotrema patialense (Digenea)

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STUDIES ON THE BEHAVIOUR AND ASSOCIATED NEURAL AND MUSCULAR ORGANIZATION OF CERCARIAE OF *TRANSVERSOTREMA PATIALENSE* (DIGENEA)

BY

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STUDIES ON THE BEHAVIOUR AND ASSOCIATED NEURAL AND MUSCULAR ORGANIZATION OF CERCARIAE OF TRANSVERSOTREMA PATIALENSE (DIGENEA)

The route of migration of cercariae of Transversotrema patialense within the host, Melanoides tuberculata has been demonstrated using serially sectioned snails at known time intervals after a stimulus which induces cercarial emergence. The cercariae migrated via the blood system from the haemocoelic spaces of the digestive gland to the sites of emergence near the rectal sinus.

The effects of experimental changes in ambient photoperiod on the periodicity of cercarial emergence indicate the presence of at least one endogenous circadian control mechanism for this behaviour. Cercariae maintained in L:D,12:12 hr. illumination cycles emerged during the dark, inversion of the photoperiod resulted in emergence reversal and continuous darkness permitted emergence to continue with circadian periodicity.

The anatomy of the nervous system of T. patialense cercariae has been described using histochemical methods for acetylcholine esterase, non-specific esterases and catecholamines. The nervous system of the cercarial head consists of two cerebral ganglia, two dorsal and ventral longitudinal nerve cords and eight pairs of outer lateral branches. The tail has a proximal and a distal nerve mass and two longitudinal nerve cords.

The effects of pharmacological agents on the swimming behaviour of cercariae have been investigated. Most of the drugs tested, whether potential transmitters, transmitter antagonists or acetylcholine esterase inhibitors, produce an inhibition of swimming activity.

The ultrastructural organization of cercarial nervous and muscular systems have been examined utilizing transmission electron microscopy. A reconstruction of the muscular system of the tail has been produced and the relationships between muscular morphology and cercarial behaviour are discussed. A range of sensory terminals, nerve fibres, nerve cell bodies, synapses and neuromuscular junctions have been characterized ultrastructurally within T. patialense cercariae. Attempts have been made to correlate these features with pharmacological and histochemical findings and to provide hypotheses concerning the nervous control of cercarial behaviour.

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CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

The family Transversotrematidae (Yamaguti, 1953) is a very homogeneous grouping of unusual digenean helminths. The transversotrematids, when compared with the majority of other digenean families, have unorthodox bodily organization, life cycle patterns and host microhabitats utilization.

All known species of transversotrematids share an unusual assemblage of morphological characters. The body is broader than it is long, the intestinal caecae have fused to form a ring, the common genital opening is situated at the anterior edge of the body and the mouth is normally on the ventral surface.

The indirect life cycles of members of the family only ever involve two hosts. The first is a freshwater, brackish water or marine gastropod, probably always in the family Thiaridae. The second is a fish. Furcocercous ocellate cercariae with arm processes at the anterior end of the tail stem swim from the snails and directly infect the fish definitive hosts. Infection of the fish hosts involves cercariae attaching to the skin surface and then moving into the sub-scale recesses of the integument. Adult worms in these recesses are utilizing a most unusual ectoparasitic host location for digeneans, one which is physico-chemically influenced by the host but which also allows direct escape of eggs. These general aspects of the biology of the family have been discussed by Anderson & Whitfield (1975) and Whitfield (1979).

The family has a distribution in the sub-tropical and tropical regions of the old world and all known species have been assigned to the genera Transversotrema (Witenberg, 1944) or Prototransversotrema (Angel, 1969). Table 1.1 lists all known transversotrematid species and indicates their geographical distribution.

Table 1.1 Distribution of the family Transversotrematidae

Species	Locality	References
Transversotrema patialense	India	Soparkar, 1924; Anantaraman, 1948; Rao & Ganapati, 1967; Murty & Rao, 1968
	Sri Lanka	Crusz, 1956; Crusz, Ratnayake & Sathananthan, 1964; Crusz & Sathananthan, 1960
	Malaysia	Sim, 1972; Betterton, 1979
	Zaire	Brien, 1954
T. soparkari	India	Pandey, 1971; Pande & Shukla, 1972
T. chackai	India	Nadakal, Mohandas & Sunderaraman, 1969; Mohandas, 1973
T. koliensis	Solomon Islands	Olivier, 1947
T. laruei	Philippines	Velasquez, 1958 and 1961
T. haasi	Red Sea	Witenberg, 1944; Overstreet, 1977
	Philippines	Velasquez, 1975
T. licinum	Australia	Manter, 1965 and 1970
T. chauhani	Lucknow	Agrawal & Singh, 1981
Prototransversotrema steeri	Australia	Angel, 1969

Soparkar (1924) provided the first description of any transversotrematid when he described a cercarial form from the freshwater snail, Melanoides tuberculata and namedit Cercaria patialensis. The genus Transversotrema was first erected by Witenberg (1944) in a description of Transversotrema haasi. He realized that this adult fluke was closely related to Cercaria patialensis.

As mentioned above, adult transversotrematids inhabit recesses under the scales of their fish hosts (Crusz, Ratnayake & Sathananthan, 1964; Manter, 1965 and 1970; Rao & Ganapati, 1967; Angel, 1969; Pande & Shukla, 1972; Sim, 1972) and this must be regarded as the normal location for adults. The findings by Velasquez (1958, 1961) of transversotrematids in the opercular cavity, gill cavity, muscles and intestine of definitive hosts have not been repeated by any other workers. The organization of the transversotrematid life cycle is considered in some detail below and is illustrated diagrammatically in Figure 1.1. The general morphological appearance of the developmental phases of the life cycles (based on that of T. patialense) is shown in Figure 1.2.

The egg-producing stage under fish scales is now generally regarded as an adult (see Angel, 1969; Manter, 1970; Pande and Shukla, 1972; Anderson & Whitfield, 1975). Earlier workers utilized the term metacercaria for this stage (Crusz & Sathananthan, 1960; Velasquez, 1961; Crusz, Ratnayake & Sathananthan, 1964). The latter terminology appears inappropriate because there is no evidence that transversotrematid ever form a metacercarial cyst and all the sub-scale inhabiting forms are sexually mature, reproducing worms.

Adults release eggs that embryonate in water (see Bundy, 1981b). A ciliated ocellate miracidium develops from the zygote within the eggs after they are released from the fish host. Sim (1972) showed that the miracidia swim rapidly and penetrate the snail body. This process must occur within a few hours of egg hatching as Bundy (1981d) has shown that the maximum

Figure 1.1 The life cycle of Transversotrema patialense

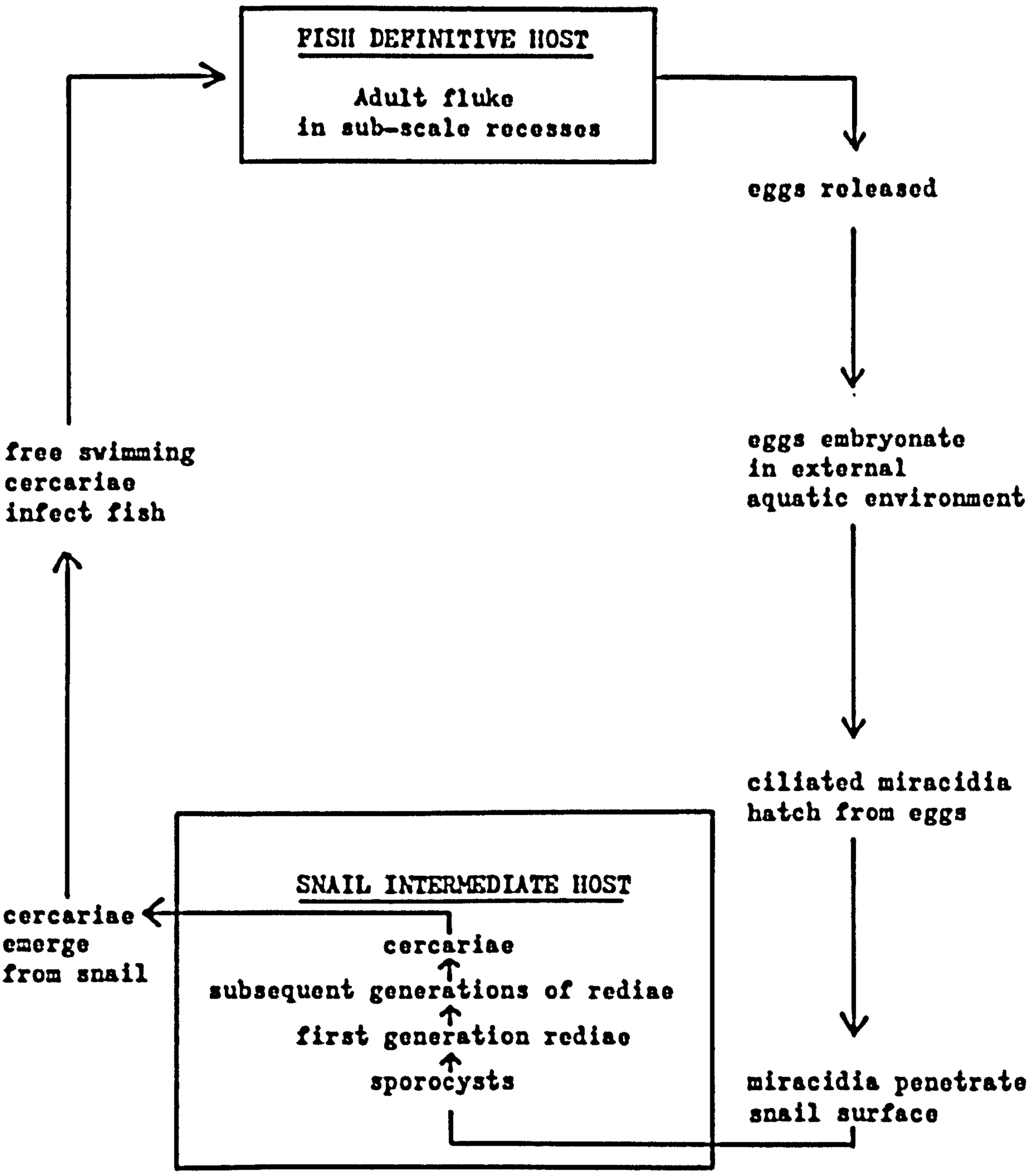

FISH DEFINITIVE HOST
Adult fluke
in sub-scale recesses
eggs released
eggs embryonate
in external
aquatic environment
ciliated miracidia
hatch from eggs
miracidia penetrate
snail surface
SNAIL INTERMEDIATE HOST
sporocysts
first generation rediae
subsequent generations of rediae
cercariae
cercariae
emerge
from snail
free swimming
cercariae
infect fish

Figure 1.2 Diagram showing stages in the life cycle of T. patialense

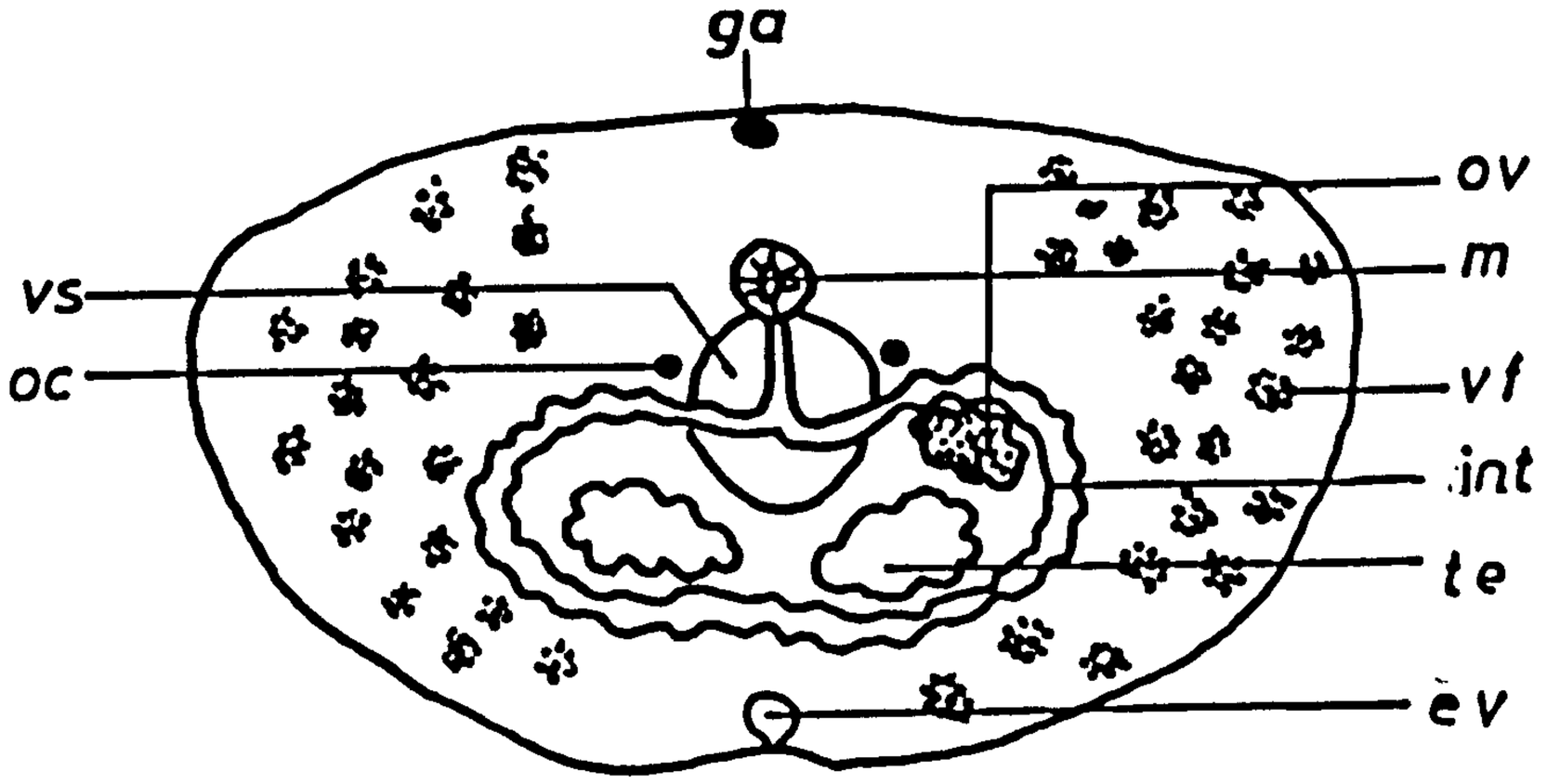
1. Adult.
   ev. excretory vesicle, ga. genital atrium, int. intestine, m. mouth, oc. ocellus, ov. ovary, te. testis, vf. vitelline follicle, vs. ventral sucker

2. Egg containing miracidium (based on an illustration in Bundy, 1979)

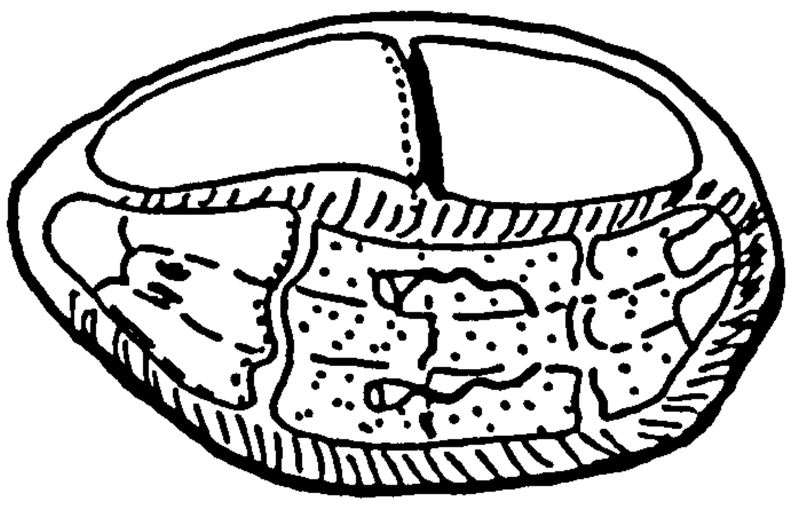
3. Young sporocyst.
   ge. germinative cell, o. ocellus from miracidial stage

4. Redia.
   gb. germ ball, i. intestine, ph. pharynx

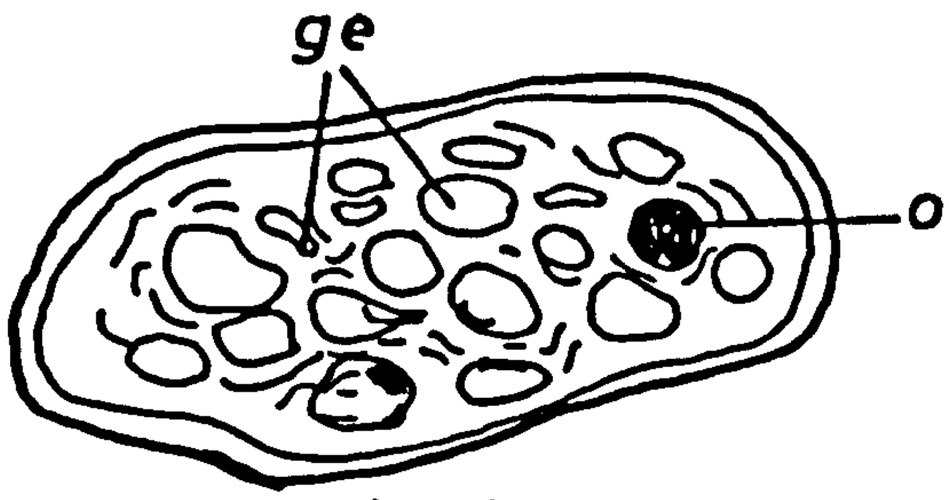
5. Cercaria.
   a. arm process, ap. adhesive pad, f. furca, ts. tail stem



(1)



(2)



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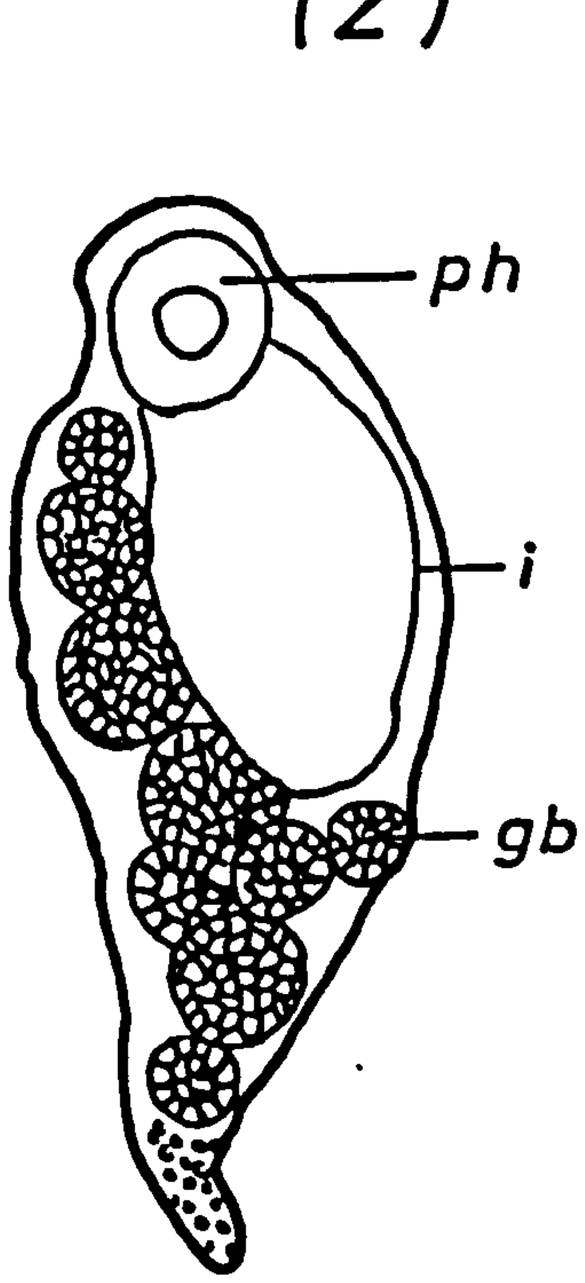
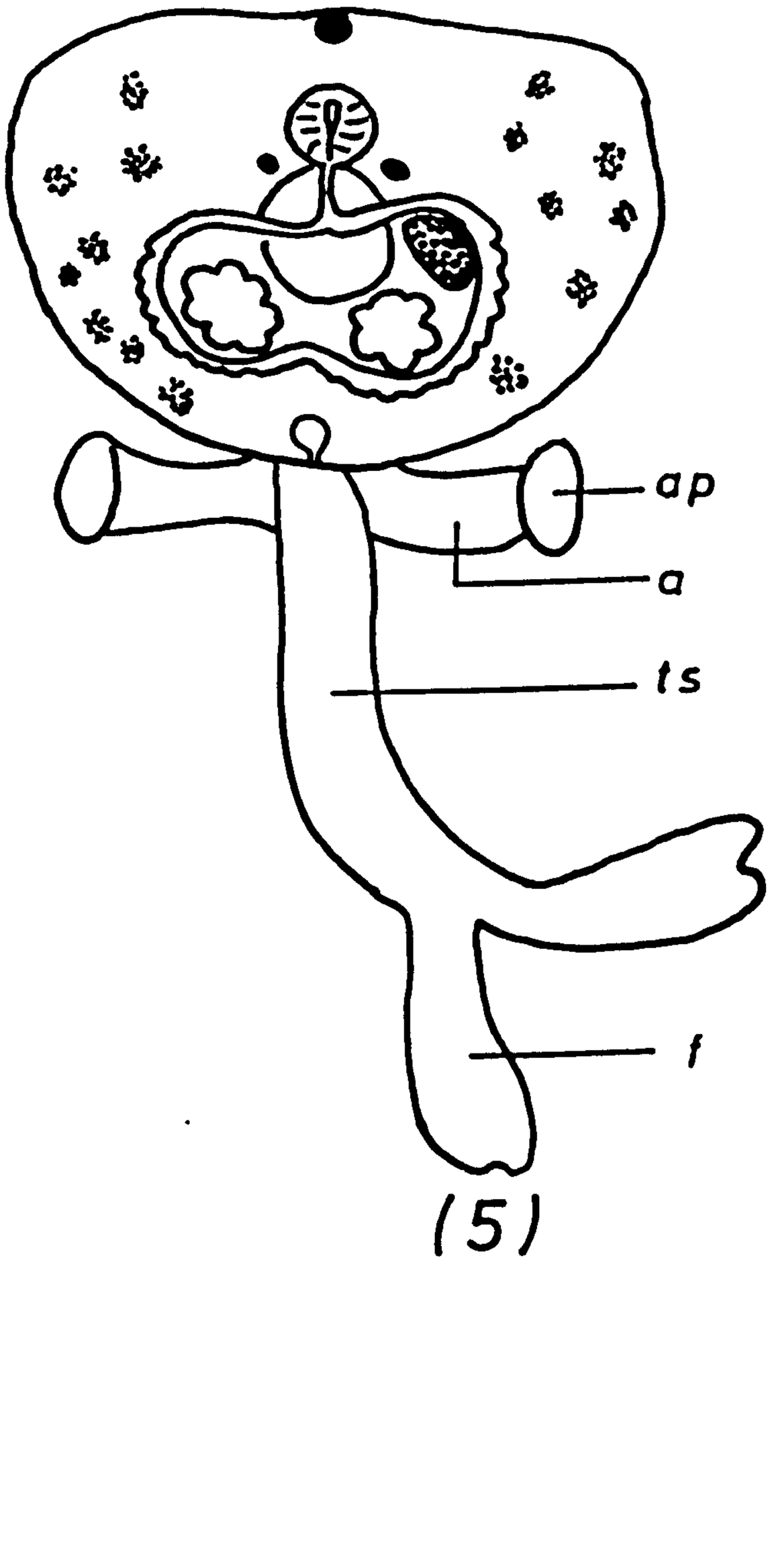


(4)



(5)

survival time of miracidia is about eight hours. Bundy (1979) has also described how each penetrating miracidium develops into a small sporocyst within the muscular tissues of the foot of the snail host (Melanoides tuberculata in the case of T. patialense). These sporocysts produce by asexual multiplication of germinal balls within the sporocyst body cavity a first generation of small rediae. These migrate to the digestive gland area of the host entering the connective tissues of this zone and the haemolymphatic spaces between the digestive gland itself and the shell. Within the first generation of rediae, germinal balls develop into daughter rediae and several daughter redial generations may be produced. Cercarial production is commenced within daughter rediae (Sim, 1972), while Bundy (1979) has demonstrated that it is quite usual for such rediae to be producing new rediae and cercariae simultaneously.

In fact, developing cercariae leave daughter rediae via a birth pore at a very early stage of development when no organogenesis has occurred. All subsequent growth and development of the cercariae occurs free in the haemocoelomic spaces of the digestive gland area (Pandey,1971; Bundy, 1979). All transversotrematid cercariae described to date possess a similar unique morphological pattern. Like the body of the adult, the cercarial head is highly flattened dorso-ventrally. The bifurcate tail bears two arm-like processes at its proximal end which themselves bear adhesive pads and specialized receptor endings (Whitfield, Anderson & Moloney, 1975). Anatomically the head of the cercariae is extremely similar to that of the adult, and mature cercariae are extremely progenetic,containing both oocytes and fully developed spermatozoa.

During the cercarial infection process the larvae attach to the final host using the arm processes to grasp its skin. The cercariae then crawl over the surface of the fish until the anterior edge of the body is pushed under the edge of one of the host scales. It then pushes its way under the scale after which the tail is shed and the head completes its development to adulthood (Whitfield et al, 1975). This development is

extremely rapid, young adults being able to produce their first eggs within 48 hours of attachment (Mills, 1980a). The development on the final host involves a moderate amount of growth and the expansion of the area of vitelline cells.

Some of the unusual features of transversotrematid biology have meant that these digeneans represent highly convenient laboratory models for particular types of helminthological experimental work. The large size of the cercariae (heads up to 534µm across) mean that they are very useful for detailed behavioural and survival studies (Anderson & Whitfield, 1975; Whitfield, Anderson & Moloney, 1975; Whitfield, Anderson & Bundy, 1977; Whitfield, Anderson & Mills, 1977; Bundy, 1981a). In addition to this advantage, the ectoparasitic final host location of these worms provides two distinct experimental benefits. Firstly, egg output can be monitored simply by filtering eggs from the water containing fish hosts, no faecal sorting being necessary (Bundy, 1981b,c). Secondly, the visibility of the flukes in their sub-scale recesses means that non-destructive population dynamical studies on adult fluke populations through time are possible (Anderson, Whitfield & Dobson, 1978 ; Anderson, Whitfield, Dobson & Keymer, 1978; Mills, Anderson & Whitfield, 1979; Mills, 1979 a,b and 1980a,b.)

The experimental advantages listed above have meant that, despite the fact that transversotrematids have no significant economic or medical importance, we probably know as much about their general biology, behaviour and population dynamics as we do about the most intensively investigated, medically significant digeneans. Within this context of available information, however, some gaps are particularly obvious, especially in the larval sectors of the life cycle. First, although much is known both about the detailed head and tail movements associated with swimming activity (Bundy, 1981a) and the temporal patterning of active swimming, dropping and resting behaviour during the aquatic existence of cercariae (Whitfield, Anderson & Bundy, 1977), next to nothing is knownabout cercarial

behaviour within the intermediate host. In particular, no information at all is available concerning the migration behaviour of cercariae within snails, which could explain how these infective larvae move from their sites of production to their points of emergence. Equally, apart from the basic fact that transversotrematid cercariae emerge in the dark (Rao & Ganapati, 1967; Anderson & Whitfield, 1975) there exists little evidence about the detailed mechanics of cercarial emergence or even its physical location within the snail host.

A second area where important information is lacking, is in respect of the physical basis of the cercarial behavioural patterns, which have already been demonstrated. A small amount is known about the tegumentary sensory organization of transversotrematid cercariae (Whitfield, Anderson & Moloney, 1975; Whitfield, 1979), but absolutely nothing has been published to date on the nervous or muscular organization of this intriguing group of helminths.

The objects of the experimental programme described in this thesis were to provide information to fill the gaps described above. The transversotrematid, Transversotrema patialense, whose life cycle is easily maintained in the laboratory, was used for these purposes. Specifically, Chapters 3 and 4 of the thesis describe the results of experiments designed to demonstrate the sites of cercarial emergence in this species, to investigate the intramolluscan migrational behaviour of cercariae and to analyse quantitatively the factors which influence cercarial emergence. Chapter 5 is concerned with the morphological description, at the light microscopical level, of the nervous system of T. patialense cercariae. Further information about the pharmacological basis of the nervous system and neuromuscular activity in these larvae are provided in Chapter 6. This reports experiments on the effects of pharmacological agents on cercarial activity. Finally, Chapters 7 and 8 provide ultrastructural descriptions of, respectively, nervous system and muscular components of T. patialense cercariae.

CHAPTER 2

General Materials and Methods

2.1 Examination of Melanoides tuberculata for infections with T. patialense

Infected snails were obtained mainly from populations in the tanks of the aquarium in the menagerie of the Zoological Society of London (London Zoo, Regents Park). Snails were isolated singly in flat-bottomed glass tubes containing 5 mls of tapwater at 26°C and placed in the dark. After one hour, the tubes were examined for the emerging furcocercariae of T. patialense.

2.2 Maintenance of the intermediate host

Infected and uninfected M. tuberculata were maintained in tanks of 13 L capacity, filled with tapwater held at between 24°C and 26°C. The tanks contained a bottom layer of fine gravel to provide a suitable substrate for these melanid burrowing snails. The food provided was fresh, clean lettuce. The snails also appeared to feed on algal film which covered the bottom substrate and the walls of the tanks.

2.3 Stimulation of cercarial emergence

The emergence of cercariae of T. patialense from infected M. tuberculata was stimulated by a reduction in light intensity following the procedure of Anderson & Whitfield (1975). Infected snails were placed in crystallizing dish containing tapwater at 26°C in the dark. Emergent cercariae were then visible under incident light against a dark background and could be removed singly using a Pasteur pipette.

2.4 Collection of intramolluscan larvae

Intramolluscan larval stages were obtained by host dissection. The shell was cut along the outside of the whorls towards the apex using fine scissors. The soft body tissues were then withdrawn from the shell and the digestive gland was isolated and teased apart using a fine needle. Developing cercariae were removed using a fine pipette and washed thoroughly in several changes of tapwater before being fixed.

2.5 Basic transmission electron microscopical technique

The basic methodology for transmission electron microscopical (TEM) studies was as follows;

Primary fixation of specimens was carried out for two hours at 4°C in 2.5% glutaraldehyde at pH 7.2 in 0.1M phosphate buffer (14 mls of 0.2M di-sodium hydrogen orthophosphate (Na_2HPO4) with 36 mls of 0.2M sodium dihydrogen orthophosphate ($NaH_2PO4.2H_2O$). Thereafter they were washed in three changes of buffer alone; each wash lasting 15 minutes at 4°C, before being post-fixed for one hour in 1% osmium tetroxide in phosphate buffer at 4°C. After post-fixation the material was washed in three 15-minute changes of phosphate buffer alone, at room temperature.

Dehydration was accomplished in a graded series of ethanol solutions, namely 30%, 50%, 70%, 90% and finally two changes of 100% ethanol. The specimens were left for 10 minutes in each part of this series.

As the embedding resin utilized and ethanol are not directly miscible, fixed specimens were transferred at room temperature through two changes of 20 minutes each of propylene oxide (1.2 epoxy-propane) which is a medium in which both resin components and ethanol are soluble.

After the final change of propylene oxide, the specimens were transferred through the following mixtures:

(a) 1:1 mixture of Spurr resin and propylene oxide for one hour at room temperature,
(b) 3:1 mixture of Spurr resin and propylene oxide for one hour at room temperature,
(c) full resin mixture overnight at room temperature,
(d) fresh resin in embedding capsules and incubated at 70°C for 24 hours.

The Spurr resin embedding medium utilized has the following composition:

10 ml ERL Vinyl cyclohexene dioxide (4206 Spurr epoxy resin).
6 ml DER 736 Diglycidyl ether of polypropylene glycol (reactive flexibilizer)
26 ml NSA Noneyl succinic anhydride (hardener)
0.4 ml S-1 (DMAE) Dimethyl amino ethanol (accelerator)

The resin blocks were trimmed and then sectioned using

glass or diamond knives on an LkB.Mk 111 ultramicrotome.

Thin sections 60nm thick were floated off onto distilled water and stretched with chloroform/xylene vapour before being picked up onto uncoated copper grids. Sections produced in this manner were stained by floating the grids on the surface of an aqueous uranyl acetate solution in a watch glass for 10 minutes at 60°C. The grids were then washed with distilled water for one minute before being stained with lead citrate (Reynolds, 1963) for 10 minutes at room temperature, followed by another distilled water wash.

Stained sections were examined with AEI.EM6B and Jeol JEM 100cx transmission electron microscopes operating at 60KV and 80KV respectively.

Specific techniques with particular significance for individual sections of experimental work will be described separately in the appropriate chapters.

CHAPTER 3

The route of emergence of the cercariae of Transversotrema patialense from Melanoides tuberculata

3.1. Introduction

Transversotrema patialense utilizes Melanoides tuberculata as its first intermediate host, a miracidium establishing an infection which eventually results in the production of numerous cercariae developing external to rediae. The mature cercariae have to leave the molluscan host to continue the life cycle; there being no encystment of metacercariae within the first intermediate host in transversotrematids. As a consequence of this, cercariae have to undertake a migration from the site of their development (in the haemocoelic spaces of the digestive gland) to the regions of emergence from the mollusc. This larval migratory aspect of digenean life cycles has received little attention in the past. Relatively few species have been studied in this respect and no detailed information exists at all for Transversotrema although some features of the intra-molluscan larval development of T. patialense have been described previously by Bundy (1979). The present investigation attempts to identify the sites of cercarial release from snails infected with T. patialense and to describe the migration route of cercariae between the digestive gland and the outside world. The study also tries to provide evidence concerning the effects of external illumination regimes on this migration behaviour.

3.2 Materials and Methods

3.2.1 Maintenance of snails.

Snails were collected and examined for infections as described in Chapter 2 (2.1).

3.2.2 Basic light microscopical techniques

Sixteen snails measuring between 1.4 and 1.6cm in shell height (12 infected and 4 uninfected) were divided into four groups each consisting of three infected and one uninfected snails. Individual snails were kept in separate 15 mls glass vials of water and maintained in a light-proof cabinet in which illumination and ambient temperature were under automatic control.

The temperature was stabilized between 24.5 and 26.5°C and a photoperiod of twelve hours of light followed by twelve hours of darkness was established in the cabinet.

Experimental snails in the cabinet were starved for a period of ten days prior to experimentation. Preliminary experiments suggested that this treatment resulted in most hard material being eliminated from the snail guts, thus aiding subsequent sectioning for light microscopy.

After this period, the snails in group I were fixed six hours after exposure to light while the snails in groups II, III and IV were fixed at each of the following times after the beginning of a dark period in the illumination regime: 10, 20 and 25 minutes.

After fixation in acetic formal alcohol solution (AFA) (5 mls glacial acetic acid and 5 mls 40% formalin and 90 mls 70% alcohol) for 24 hours the snails were decalcified in Gooding and Stewart's formic acid mixture (Carleton & Leach, 1938) for 24 hours, washed in running water for three hours, dehydrated in a graded ethanol series (30%, 70%, 90%, 100%; two changes, two hours each) and cleared in toluene (two changes, one hour each). After two changes of 30 minutes in 58°C paraffin wax, the snails were each embedded in a watch glass containing fresh hot paraffin wax. The snails were cut sagittally with respect to their head regions at a thickness of 7 µm and the serial sections so obtained mounted on slides which were dried and then stained with Ehrlich haematoxylin and Eosin Y.

3.2.3 Snail dissection

A knowledge of the anatomy of the snail is essential to understanding of the migration route of cercariae within it.

To aid in the interpretation of serial sections some uninfected molluscs were dissected. Dissection was carried out after fixation in AFA. To remove the body from the shell, the latter was carefully broken and the columella muscle dislodged from it. Dissection was carried out in a small petri dish with the help of fine scissors and forceps under a dissecting microscope. The previous descriptions of Berry (1974) and Yousif (1975) proved helpful in the interpretation of dissected preparations.

3.2.4 Quantitative analysis of the positions of rediae and cercariae in serial sections

Numerical information on the relative distribution of rediae and cercariae (counted as heads) in different snail tissues was obtained in different ways in the various parts of the hosts' anatomy. In all tissues and organs other than the digestive gland, parasitic larvae were present at low enough densities for all larvae to be identified and counted individually. Previous measurements on the maximum dimensions of larvae within hosts had revealed that the average length of a redia was about 290 μm and the average width of a cercarial head about 360 μm. Given these dimensions, it was assumed that in tissues other than the digestive gland a monitoring of every seventh 7 μm section would reveal all the parasites.

In the digestive glands of infected snails, rediae and developing cercariae are often very densely packed together. In these circumstances it was necessary to utilize a sampling method to estimate larval numbers and the spacings between counted sections utilized were chosen so as to ensure that only very infrequently would a single larva be counted more than once. In the digestive gland, rediae were counted in every 42nd section and cercarial heads every 84th section. Other developing stages encountered in the digestive gland which would not be identified unambiguously as rediae or cercariae were ignored.

3.2.5 Photomicrography

Photomicrographs of larvae *in situ* within hosts were obtained from the serial sections on a Carl Zeiss photomicroscope using Ilford FP4 135 film (125 ASA) and Ilford HP5 (400 ASA).

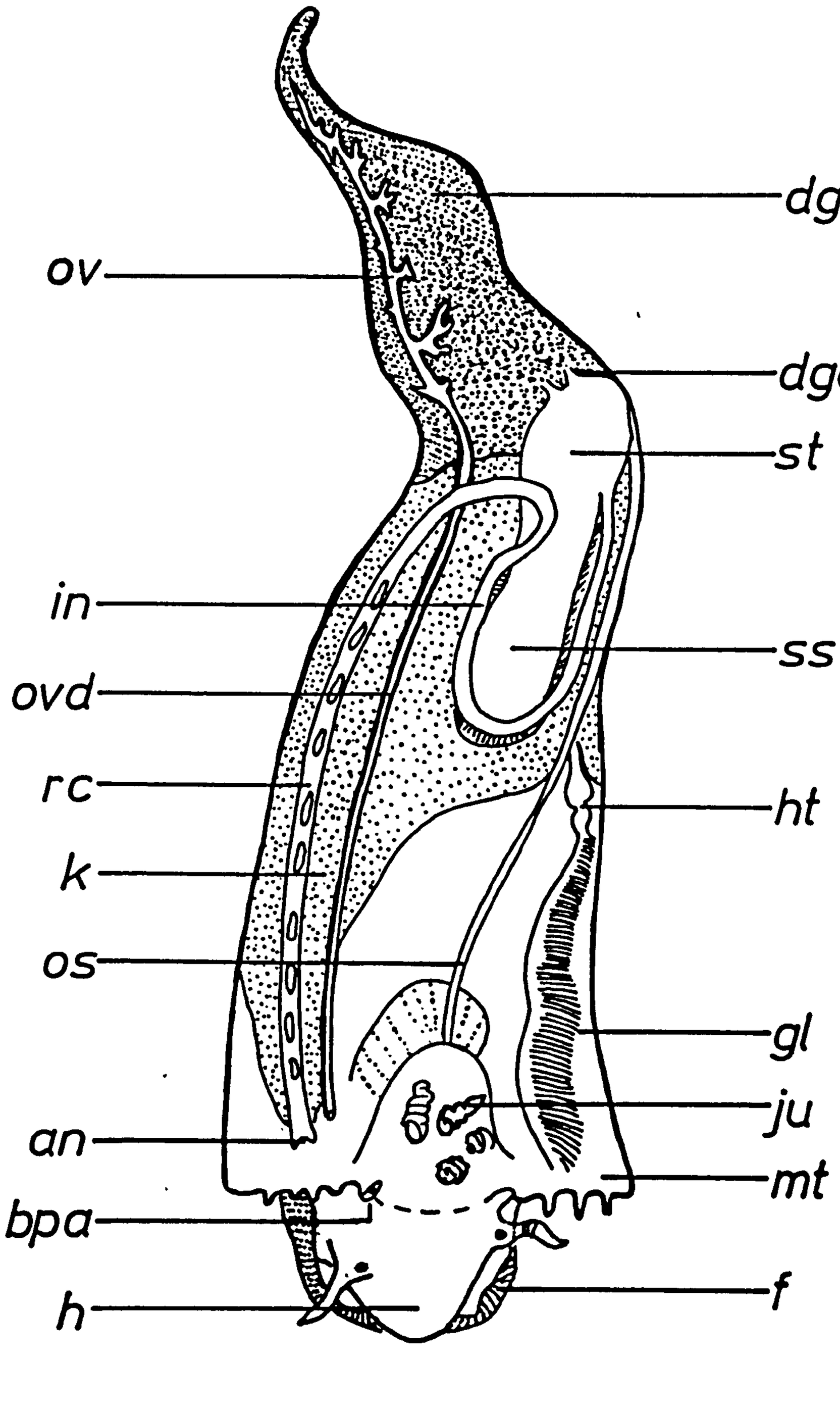
3.3 Results

3.3.1 Macroscopic anatomy of uninfected Melanoides

Upon removal of the shell, the body of Melanoides shows a division into three distinct regions, the head, foot and visceral mass. On opening the mantle with a mid-dorsal longitudinal incision (Figure 3.1), a prominent gill or ctenidium appears on the left and the rectum on the right. The mouth opens into a buccal

Figure 3.1 Melanoides tuberculata with mantle opened mid-dorsally.

Key: an, anus; bpa, brood-pouch aperture; dg, digestive gland; dgd, digestive gland ducts; f, foot; gl, gill; h, head; ht, heart; in, intestine; Ju, Juvenile; k, kidney; mt, mantle; os: oesophagus; ov: ovary; ovd: oviduct ; rc, rectum; ss, style sac; st, stomach.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mass to which the oesophagus is attached posterially. Two long, tubular salivary glands open dorsally into the buccal mass. The oesophagus leads to the posterior part of the stomach which also receives two ducts from the digestive glands. A style sac appears as a bulbous extension of the stomach. The intestine arises from the middle region of the stomach coils over the stomach and then leads along the right wall of the mantle to the rectum and anus. A flattened whitish kidney with triangular outline lies dorsal to the stomach, and within the roof of the mantle cavity. The heart lies to the left behind the mantle cavity. A branched tubular ovary can be recognized partially surrounding the digestive gland and a simple narrow oviduct leads from it and opens just behind the anus. Several young snails can usually be seen in the brood pouch which is positioned in the dorsum of the head and opens on the right side by a relatively wide opening located behind the base of the right tentacle.

3.3.2 Locations of T. patialense larvae in serial sections

During the life cycle of Transversotrema patialense (see Bundy, 1979) the miracidium penetrates the muscular foot of the snail host and transforms to become a mother sporocyst. The germ balls within the sporocyst undergo an asexual reproduction giving rise to next larval stage, the rediae. The rediae migrate to the digestive gland and after several generations of daughter rediae give rise to furcocercous cercariae which develop free in the haemocoelomic space of the digestive gland of the snail.

As was expected from this developmental scheme, analysis of larvae in snail sections in this study revealed large numbers of rediae and cercariae in the haemocoelomic spaces around the outer margin of the digestive gland whorls, the site where most cercarial and redial production within rediae appears to occur. Rediae were also found in the visceral, kidney, rectal and mantle sinuses, visceral connective tissue, gill, head and foot. Some were presumably rediae moving from

areas like the foot to the digestive gland.

The route of cercarial migration within the snail from the digestive gland to the outside world has been investigated by recording the number of cercariae in the different tissues of the snails before and immediately after the initiation of a dark period in the illumination regime. Previous studies (Whitfield, 1979) have shown that almost all cercarial output occurs in the dark and it was hoped that at the time around the beginning of this period cercariae would be moving from the digestive gland to the points of emergence. Table 3.1 lists the positions of rediae and cercariae in all four experimental groups of snails.

In snails fixed during the light period, the cercariae were found exclusively in the digestive gland, while the rediae were found in haemocoelomic spaces in the digestive gland, visceral connective tissue, head, foot, as well as the visceral, kidney, rectal and mantle sinuses.

The distribution of rediae and cercariae along the snail body fixed after 10 minutes exposure to darkness revealed that the concentration of cercariae in the digestive gland become dispersed with a few cercariae being found in the visceral, kidney, rectal and mantle sinuses (Plates 3.1, 3.3, 3.4, 3.5A). The rediae were found in these same sinuses as well as in the head and foot (Plate 3.2).

After 20 minutes exposure to darkness, there was an apparent progressive increase in the number of cercariae recovered in the sinus locations mentioned above and a particularly dramatic increase in the rectal sinus. The redial distribution remains unchanged with no rediae being found in the blood spaces of the visceral connective tissue.

The distribution of cercariae after 25 minutes exposure to darkness showed the same general pattern as shown after 20 minutes except that cercariae were also visible in the blood spaces of the gill and mantle cavity (Plate 3.5B). An even more marked increase in cercarial number was encountered in the rectal sinus. Again there appeared to be no change in redial distribution

Table 3.1 The distribution of rediae and cercariae of T. patialense in different tissues of the infected snail host, M. tuberculata and at different times during a 12:12,D:L illumination regime

Tissues & organs	Group I: Fixed during the light period								Group II: Fixed after 10 mins. exposure to darkness								Group III: Fixed after 20 mins. exposure to darkness								Group IV: Fixed after 25 mins.exposure to darkness							
	No. of rediae & cercariae per individual snail						Total no. of rediae & cercariae per 3 snails		No. of rediae & cercariae per individual snail						Total no. rediae & cercariae per 3 snails		No. of rediae & cercariae per individual snail						Total no. rediae & cercariae per 3 snails		No. of rediae & cercariae per individual snail						Total no. rediae & cercariae per 3 snails	
	1		2		3				1		2		3				1		2		3				1		2		3			
	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R
Digestive gland	89	49	44	43	21	21	154	113	18	23	39	20	18	21	75	61	55	18	20	14	48	20	123	52	40	18	171	37	29	17	240	72
Visceral sinus		7				6		13		3	2	1			2	4	6	5					6	5	7		1		4	1	12	1
Visceral connective tissue		7		1		3		11																				1				1
Kidney sinus				7		5		12	1			1			1	1	7	4					7	4	11		1	3	4	1	16	4
Rectal sinus		2		1				3	1	2					1	2	21	17	4	3	2		27	20	41		23	1	8	1	72	2
Gill				3		1		4										5						5	1			1		1	1	2
Mantle sinus		6		2		7		15	1	1					1	1	1	14		1		2	1	17	5	3	5			2	10	5
Mantle cavity																									1						1	
Head		12		8		10		30		5		3		4		12		8				2		10		12		4		1		17
Foot		20		26		5		51		16		10		1		27		14				6		20				17		13		30

C: Cercariae R: Rediae

It should be noted (see Section 3.2.4) that different sampling techniques are utilized in counting larvae in the digestive gland from those employed in respect of other tissues.

compared with the situation in the light. Figure 3.2 summarizes the information concerning movements of cercariae into sinuses outside the digestive gland.

Summarizing these changes in the position of cercariae it appears that in the light all the cercariae within an infected snail are restricted to the blood spaces of the digestive gland. This is so despite the vascular system links between these spaces and the rest of the circulatory system of the host. Between 10 and 25 minutes after experiencing the beginning of a dark period, this cercarial distribution pattern changes drastically and progressively. More and more cercariae are seen in almost all the circulatory sinuses (see Table 3.1, Figure 3.2), but by far the greatest proportion of these migrating larvae are found in the rectal sinus, a pattern that is particularly clear after 25 minutes in the dark.

At 10, 20 and 25 minutes into the dark period, the relative numbers of cercariae in the non-digestive gland sinuses are in the proportions 1.67:13.67:37.33. At 25 minutes the trend in numbers within all the sinuses appears still to be increasing. It should be emphasized that while these positional changes are occurring the majority of a host's cercariae are still within the digestive gland. To attempt to interpret these changing locations of cercariae in the early minutes of a dark period it is necessary to understand the blood system connections, and blood flow directions that exist in a grastropod such as Melanoides. Figure 3.3 illustrates these features diagrammatically being based on the information available in Morton (1958). No detailed accounts appear to exist of the blood system anatomy of this species and it has been assumed that it conforms to the relatively conservative gastropod pattern. The large spaces within the body are haemocoelic. The head, foot and viscera are supplied directly with blood by two arteries which arise from the ventricle of the host. The blood seeps from the arteries into pseudovascular spaces in the connective tissue. In gastropods, peripheral blood from the head, foot and much of the mantle is returned into a central space known as the cephalopedal sinus. From the

Figure 3.2 Time course of increases in T. patialense cercarial numbers in M. tuberculata sinuses outside the digestive gland before and after the beginning of a dark period.
(Each data point represents cercarial numbers summed from three experimental snails.)

•———• represents number of cercariae in the kidney sinus

•- - - • represents number of cercariae in the visceral sinus

•.....• represents number of cercariae in the mantle sinus

•.....• represents number of cercariae in the rectal sinus

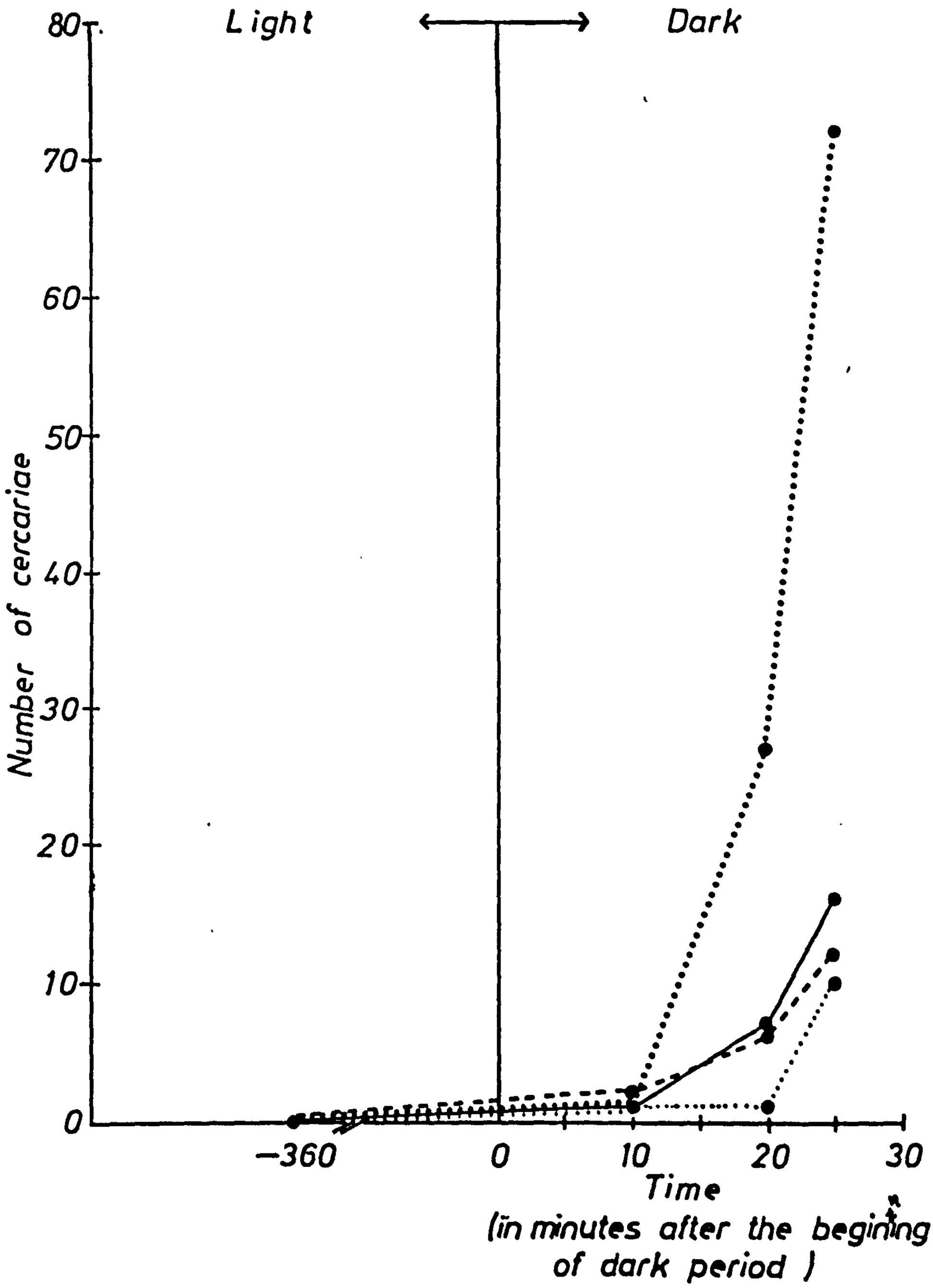
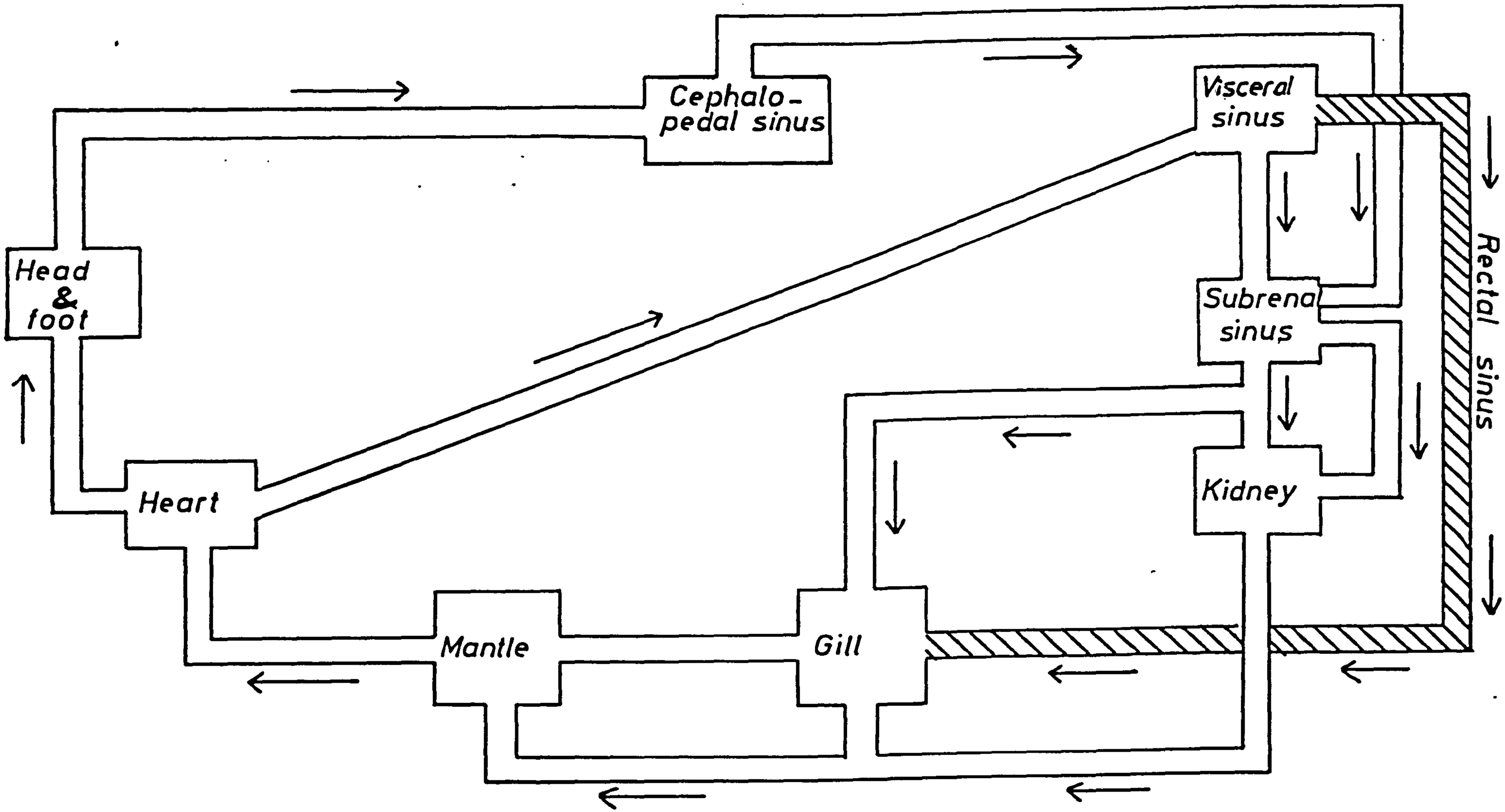

Light
Dark
80
70
60
50
40
30
20
10
0
Number of cercariae
−360
0
10
20
30
Time
(in minutes after the beginning
of dark period)

Figure 3.3 Diagram showing the generalized structure of the circulatory system of mollusc like Melanoides.
→ Direction of blood flow (based on information in Morton, 1958)


Cephalo-
pedal sinus
Visceral
sinus
Rectal sinus
Head
&
foot
Subrenal
sinus
Heart
Kidney
Mantle
Gill

visceral mass it is brought back to the visceral sinus. Both these spaces discharge into the subrenal sinus which lies near the columella muscle at the base of the visceral mass. From here it is distributed in varying proportions to the respiratory organs and the kidneys before returning to the heart. There is an extensive renal portal system into which all or part of the venous blood may go, passing thence either to the gill or directly to the auricle. By an alternative route, the rectal sinus, blood may be sent from the visceral sinus along the mantle roof straight to the gill without passing through the kidney.

Clearly, from the visceral haemocoel, blood can follow three distinct pathways to the mantle (Figure 3.3) and thence to the heart:

a) via the rectal sinus passing through the gill;

b) via the subrenal sinus to the kidney and thence along the renal portal vein;

c) via the subrenal sinus passing through the kidney and gill or the gill alone.

Oxygenated blood from the mantle passes into the heart and then into the aorta which gives off an anterior and a posterior branch which supply all organs of the body.

Coupling the known positions of cercariae with this information concerning blood flow provides a confusingly large number of alternative routes by which cercariae might accomplish the migration from the digestive gland to the outside world. The actual site of emergence was not directly observed in this study, but it is almost certainly significant that the first cercaria to be seen outside the body after the commencement of the dark period in these experiments was in the mantle cavity. This location perhaps suggests that the route of exit must pass through the body wall of the snail somewhere on the upper surface of the mantle cavity. Only two locations appear to provide cercariae with the possibility of a thin tissue barrier between the vascular system lumen and the cavity of the mantle or an adjacent region. These are the mantle sinus and the rectal sinus, both of which have been shown to contain cercariae from 10 minutes into a dark period onwards.

Cercariae could possibly leave the snail through the mantle edge or near the anus from the mantle sinus, or through the inner or outer wall of the mantle via the rectal sinus. Although cercariae were never seen in the rectal lumen itself, there remains a possibility that cercariae could exit from the rectal sinus by passing into the rectum and then out through the anus.

In several cases although emergence could not be directly seen in a serial section, cercariae were seen in sinus lumens separated from the outside world by tissue barriers that appear to be pathologically narrowed. In Plate 3.5 for instance, two cercariae are visible immediately beneath a tissue layer that appears to consist of little more than a single stretched layer of epithelial cells.

The fact that cercariae are never (see Table 3.1) found in the tissues of the head or foot probably means that having left the region of the digestive gland they are unable to make a complete circuit of the blood system via the heart. (It is difficult, however, to exclude the possibility, that some may pass through the heart and regain entry to the visceral sinus via the visceral artery.) If the cercariae do leave the snail body via the mantle or rectal sinuses, and they do not pass through the heart, one is dealing essentially with a unidirectional flow of cercariae from the digestive gland and visceral sinus to the sinuses and organs of the mantle. The proportional distribution of cercariae in these locations suggest that the greatest proportion must pass from the visceral sinus to the rectal sinus. The presence of any cercariae at all in the subrenal and kidney sinuses means that some cercariae leave the visceral sinus via the subrenal route. Cercariae seen in the gill and mantle sinus could have reached these locations either via the rectal sinus or the renal portal system. The disproportional accumulation of cercariae in the rectal sinus probably suggests that their onward movement from this site is somehow being retarded. It is impossible at present to know whether such retardation of movement is due to a restriction of the diameter of the vessels at this point or a behavioural response on the part of the cercariae themselves.

Equally, the movement of cercariae from the digestive gland to the mantle cavity sinus is an unknown mixture of passive and active movement.

Rediae showed no clear patterns of changing distribution around the time of the transition from the light to a dark period. Although many rediae are found in haemocoelomic spaces like cercariae, others are located in solid tissues such as those of the head and foot.

3.4 Discussion

The results of the investigation described above indicate that the migration of T. patialense cercariae within their snail host takes place via the blood system.

A number of investigations by other workers on other digenean species concerning the route of cercarial migration have indicated that cercariae may utilize a variety of routes of migration within the snail host to the site of escape to the exterior.

The migration of cercariae within the snail host via the blood system has been described for Schistosoma mansoni (see Duke, 1952; Richard, 1961); Alaria arisaemoides; Alaria canis (see Pearson, 1956); Strigea elegans and an unidentified schistosome (see Pearson, 1959); Neodiplostomum intermedium (see Pearson, 1961) and Cercaria X (see Probert & Erasmus, 1965). Migrations of sporocysts and rediae (both containing cercariae) via the circulatory system to the site of cercarial release have been reported in Cercaria vaullegeardi (Matthews, 1980) and Cercaria calliostomae (Matthews, 1982).

Other routes have also been described. The migration of cercariae via spaces interpreted as lymphatic channels to the site of emergence has been reported in Fasciola hepatica (Kendall & McCllough, 1951) and Vasotrema robustum (Wall, 1951). Lutz (1919) found that cercariae of Schistosoma mansoni escaped via the intestinal canal and Campbell & Todd (1956) found a similar condition in Fascioloides magna. Schistosoma mansoni cercariae have been demonstrated within the eggs of the snail host by Brumpt (1941), who concluded that the female genital tubes must represent one of the routes of emergence.

In T. patialense the present findings indicate that the migration of cercariae from the digestive gland to the region of emergence is via the main venous vessels, particularly the rectal sinus with the final accumulation of cercariae in several of the blood vessels of the mantle wall.

The suggestion that the rectal sinus constitutes the main pathway along which migrating cercariae travel finds support in the previous findings on other digenean species by Duke (1952), Pearson (1956; 1959; 1961), Richard (1961) and Probert & Erasmus (1965). Kendall & McCllough (1951) have also demonstrated migration via the rectal sinus in Fasciola hepatica but by the way of the lymphatic channels.

Cercarial migration was initiated by the dark period of an L:D,12:12 photoperiod since no cercariae were found outside the digestive gland area in snails fixed during the light period. The migration started within the first 10 minutes of the dark period and continued at an increasing rate over the next 15 minutes. Other direct studies on cercarial emergence (see Chapter 4) will reveal that the peak of such emergence into the external environment occurs between 30 and 45 minutes after the beginning of the dark period.

T. patialense cercariae, produced within rediae in the digestive gland are either already within the haemocoelomic spaces of this organ or can reach such sites by breaking through small discontinuities in the walls of these spaces within the digestive gland itself. By either means the cercariae will then be topologically within the blood system and capable of moving to the visceral sinus without any additional breaking of tissue barriers. As described in the Results section, it is possible that the cercariae of T. patialense emerge to the exterior via the thin body wall over the rectal or mantle sinuses or into the rectum itself or by both routes. Given this assumption, it is reasonable to assume that localized muscular activity associated with the rectum might be implicated in moving cercariae within these sinuses, or even associated with emergence itself.

Emergence of cercariae through an area of body wall immediately contiguous with the anus of the snail has been demonstrated by Kendall & McCllough (1951) for Fasciola hepatica. They concluded that the cercariae accumulated in the space surrounding the distal part of the gut and that the actual emergence was predominantly passive with the passage through the body wall to the exterior being aided by the muscular contraction of the mantle and pneumostome of the mollusc.

Other methods whereby cercariae escaped from the tissues of the mollusc host have been reported by other workers using a number of digenean species. Work by Faust & Meleney (1924) on Schistosoma japonicum and by Faust & Hoffman (1934) on Schistosoma mansoni, for instance, suggests that some emergence of cercariae in these species takes place by rupture of the tunica propria, the body wall covering of the digestive gland area. More recently, however, Duke (1952) utilized serial sections of infected snail hosts and found that Schistosoma mansoni cercariae arriving via the peri-rectal spaces at the pseudobranch and collar, penetrated the integument there and emerged to the exterior through an escape pore. Active escape from the blood vessels through fixed points on the mantle wall, collar and pseudobranch has also been described for Alaria arisaemoides and Alaria canis (see Pearson, 1956); Strigea elegans (see Pearson, 1959); Neodiplostomum intermedium (see Pearson, 1961) and Cercaria X (see Probert & Erasmus, 1965). In the case of Dicrocoeloides petiolatum active escape of daughter sporocysts, containing cercariae occurs through the pneumostome (see Timon-David, 1960).

Summarizing the results from a range of different digenean families, it seems likely that in most cases cercarial migration and emergence are complex processes involving activity on the part of both the cercariae and the snail in order to bring them about in a patterned way. It would further appear that at least in some cases the points of emergence from a particular snail seem to be relatively fixed. Once temporary escape pores are formed, presumably by activities of cercariae, subsequent cercariae may utilize the same openings.

When one considers the migrational and emergence behaviour of T. patialense cercariae a number of interesting questions arise. The cercariae appear to be able to both break into blood vessels

from the digestive gland and to break out from them through the body wall of the mantle to enter the outside world. What means do the larvae possess for breaking these tissue barriers?

They have no specialized gland cells or stylets which normally characterize cercariae that can penetrate tissues and the flattened head shape is apparently not an efficient one for penetrating any substratum. It remains a possibility that the fully developed gut of the cercaria with synthetic gastrodermal cells (see Bundy, 1979) might be able to produce lytic secretions that could be used in an extracorporeal fashion to achieve tissue break down. Images like that in Plate 3.5 showing an abnormally thin body wall adjacent to cercariae might represent the effects of such secretions.

The fact that cercariae have never been observed in this study actually in the process of passing through an opening in the body wall is almost certainly due to the rapidity of this phenomenon. Given the descriptions of other species of digenean it appears at least plausible that exit holes made by one cercaria might be used by subsequent larvae. As they are emerging from the lumens of blood vessels, escaping haemocoelomic fluid might assist this process.

Cercarial movement through the blood vessels and sinuses from the digestive gland area to the mantle could be passive or active. Both methods appear possible because in the case of Neodiplostomum intermedium, Pearson (1961) has observed cercariae crawling along the blood vessels of the snail host aided by blood flow.

A final question concerns the temporal pattern of the movement of cercariae from the digestive gland area. This study has shown that it begins within 10 minutes of the beginning of a dark period in an illumination regime. The nature of the immediate stimulus that brings it about, however, remains unclear. It is not certain, for instance, whether it is a direct behavioural response on the part of the larvae or an effect mediated by the snail, due for instance to a change in blood flow or blood vessel calibre.

Once the movement begins, it seems possible that the general direction of the cercarial migration is dictated entirely by the pattern of blood flow. Even without active movements by the cercariae they would reach the locations in the blood vessels and sinuses of the mantle area in which this study has found them (see Figure 3.2).

As has been described above, rediae of T. patialense do not show the pattern of migration in time that cercariae do. Bundy (1979) has shown that the distribution of intramolluscan stages during their development changed markedly with time after the infection established. He found that the time scale of the development is as follows: seven days after the penetration of the miracidia mother sporocysts within the muscular tissue of the foot were recovered. 33 ± 10 days post infection, large and very small rediae were observed in the cardiac haemocoelomic space and within the digestive gland. At 68 ± 10 days post infection a variety of sizes of rediae were found in the digestive gland. Immature cercariae were also present. At 102.5 ± 10 days and 183 ± 13 days a variety of sizes of rediae and cercariae were present in the digestive gland. The distribution of rediae described in this study are fully comparable with an overlapping series of waves of redial colonization as described by Bundy.

The detailed redial distributions described in this study enable some of Bundy's general findings to be explained. It appears that following miracidial penetration and transformation to mother sporocysts, the freshly liberated redia through its own active peristaltic movement and assistance of the circulatory blood, enters the cephalopedal sinus and reaches the cardiac haemocoel spaces by way of the ctenidial or renal circulation,then with the oxygenated blood via the posterior branch of the aorta, the rediae arrive at the digestive gland. The few rediae that were recovered in the loose connective tissue surrounding the visceral haemocoel probably represent young rediae, that having reached the visceral sinus by the route described above, were migrating towards the adjacent digestive gland lobules.

The present discussion has concentrated on a consideration of the migration of rediae and cercariae of T. patialense within the snail host but attention must also be given to the effect of the parasite on the snail although little evidence for direct histopathology related to the T. patialense larvae has been found in this study. No young snails were observed in the brood pouch of infected snails. It seems very likely that this lack of reproductive activity may be due to the larvae of T. patialense within the snails which resulted in an inhibition or reduction in the

reproductive capacity of the hosts.

Bundy (1979) studied the development of intramolluscan stage of T. patialense and noted that when approximately 40 rediae were the only inhabitants of the digestive gland (up to 68 days PPI) the snail was able to reproduce. Once intramolluscan cercarial development was initiated there was a decline in the reproductive efficiency. Whitfield (1982) has described a similar decline in the activity of the female reproductive system of Melanoides infected with T. patialense. He has also demonstrated that the otherwise parthenogenetic female snails develop transient testes during the infection.

These changes in the reproductive ability of infected Melanoides may be produced directly, by disruptive changes to reproductive systems, or indirectly, by reduction of nutrient supplies to gonads or the perturbation of host hormonal control.
(See Wright, 1966, for a review of a digenean-induced pathology in snails.)

CHAPTER 4

The emergence rhythm of *Transversotrema patialense* cercariae

4.1 Introduction

A crucial aspect of cercarial behaviour in most digenean species is the pattern of activity which results in cercarial emergence from the first intermediate molluscan host. Despite the fact that this phase of the digenean life cycle has such great importance in transmission dynamics, little is known about the mechanisms which are responsible for particular patterns of emergence. It is often not clear, for instance, to what extent emergence is, an active escape on the part of the cercariae, a release allowed by behavioural or physiological changes on the part of the host, or a combination of both types of phenomena.

External environmental conditions certainly influence emergence behaviour and the cyclical nature of emergence patterning in the absence of potent cyclical external stimuli has lead many workers to conclude that endogenous biological clock mechanisms must also be involved (Asch, 1972; Wagenbach & Alldredge, 1974).

In this chapter the results of a series of experiments on the emergence behaviour of T. patialense cercariae are recorded. The experiments have been designed to provide as much information as possible on the mechanisms which generate emergence, with particular reference to the role that endogenous "biological clocks" might play in this emergence.

4.2 Materials and Methods

4.2.1 Acclimatization and other general procedures

In all experiments concerned with the effect of varying light-dark regimes on the emergence of T. patialense cercariae from their molluscan hosts, a set of standardized acclimatization and related procedures were used. All the experiments were carried out in a light-proof cabinet in which illumination and ambient temperature were under automatic control.

The cabinet was equipped with one 15 watt "daylight" fluorescent tube which when in operation provided an illumination intensity of 1600 - 2400 lux close to the snails. In all experiments the ambient temperature was held between 24.5 and 26.5^{o}C and a repeating 12 hours light, 12 hours darkness illumination regime (L:D, 12:12) utilized initially.

Infected snails were kept in the experimental cabinet in separate 15 ml glass vials and provided with clean lettuce as food for a period of four days at the beginning of each experiment in order to acclimatize them to the initial experimental conditions. During experimental periods monitoring of cercarial emergence was carried out by the transfer of snails at appropriate intervals into new vials of water previously equilibrated to cabinet temperature conditions. The cercariae in removed vials were immobilized by the addition of 2 ml of 4% formalin and counted directly. In some experiments the width of fixed and flattened cercarial heads were measured using a previously calibrated eyepiece micrometer.

Access to the cabinet for vial transfers was accomplished via a light-proof entrance chamber. During light periods no appreciable change in illumination occurred during transfers. During dark periods it was necessary to use approximately 30 seconds of very low intensity photographic safe lighting (Kodak 1A filter) to carry out transfers.

For all experiments, before snails were placed in the cabinet for pre-experiment acclimatization, they had been kept in a natural LD regime for at least 10 days previously. For each of the experiments described below, this natural LD period is recorded in brackets at the beginning of the appropriate section.

4.2.2 Experiment 1

L:D, 12:12 (12 hrs light; 12 hrs dark) regime (natural L:D, 16.63:7.37)

This experiment was performed with a view to discovering when, in a general way, cercariae emerged during cyclically variable illumination conditions.

Eleven infected snails were observed over a period of five consecutive days, being exposed to alternating periods of 12 hours darkness (from 19.30 to 7.30) and 12 hours light (from 7.30 to 19.30). Determination of the numbers of cercariae emerging per snail were made over 24 hour time intervals on day 1, day 2 and day 4, while on day 3 and day 5, vial transfers were carried out every three hours. During the entire extent of this experiment head widths of all the cercariae which emerged were measured. At the end of the experiment the wet weights of the experimental snails were determined.

4.2.3 Experiment 2

L:D, 12:12 (12 hrs light; 12 hrs dark) regime with sampling at short intervals (natural L:D, 16.58: 7.42)

Experiment 1 was repeated using vial transfers at shorter intervals of time to assess with a higher temporal resolution the pattern of cercarial emergence. On days 3 and 5 counts of cercariae were made every 15 minutes, starting one hour before the beginning of the dark period (i.e. at 18.30) and continuing for a total of four hours.

4.2.4 Experiment 3

L:D, 12:12 regime reversal (natural L:D, 15.03:8.97)

In this experiment the snails were fed during the first five days. After nine days of L:D, 12:12 regime, the light cycle was reversed during days 10, 11 and 12. This reversal was made at 17.30 hrs at what should have been the beginning of the dark period of day 10 of the experiment, so that two 12 hr light periods came in succession followed by alternating 12 hr dark and light periods.

Observation on the number of cercariae released were made every 24 hrs in the first nine days while in day 10 counts of cercariae were made at two hourly intervals starting at 16.30 hrs and continuing throughout day 11 and day 12 until 16.30 hrs. The next sample was taken at 10.30 hrs the following day.

4.2.5 Experiment 4

Regime change from L:D, 12:12 to LL (continuous light) (natural L:D, 10.35:13.65)

This experiment was performed to discover whether cercariae would emerge if the snails were kept in continuous light, for a period of several consecutive days after previous exposure to L:D, 12:12. It was also hoped to discover the temporal pattern of such output if it occurred. 17 infected snails were exposed for two days to continuous light L:L,12:12 after 10 days exposure to normal L:D, 12:12. During this initial period illumination began at 10 hrs and ceased at 22hrs. Collection of the emitted cercariae were made at 48 hr time intervals during the first 10 days, while on days 11 and 12 vial transfers were carried out every two hours.

4.2.6 Experiment 5

Regime change from L:D, 12:12 to DD (continuous darkness) (natural L:D, 14.02:9.98)

This experiment was carried out to determine whether an innate emergence pattern was displayed when infected snails were placed in conditions of continuous darkness.

11 infected snails were initially exposed to an L:D, 12:12 regime for eight consecutive days. They were then subjected to continuous darkness for a further 10 consecutive days. The light period began at 5.30 hrs and ended at 17.30 hrs at the beginning of the experiment. The number of cercariae released were determined at different time intervals throughout the experiments. The details of changing cercarial collection period through this experiment are shown in Table 4.7. The snails were supplied with an excess of food for the first four days.

4.2.7 Experiment 6

Faecal production under L:D, 12:12 (12 hrs light; 12 hrs dark) regime (natural L:D, 16.50:7.50)

This experiment was performed with a view to ascertaining when faeces were produced during cyclically variable illumination conditions and whether there was any relationship between such a pattern and the pattern of cercarial emergence.

20 snails of approximately equal weight (mean, 325 mg wet weight) (10 infected and 10 uninfected) were divided into two pairs of groups each consisting of five infected and five uninfected snails and maintained in alternating periods of 12 hrs darkness (from 22.00 to 10 hrs) and 12 hrs light (from 10.00 to 22 hrs) for 11 consecutive days in separate 15 ml vials and fed daily with an excess of clean lettuce. After five days of normal feeding, all feeding was stopped in one 5-snail group of uninfected and infected hosts for six days. Faecal production was determined at two hourly intervals during day 11.

4.3 Results

4.3.1 Experiment 1; L:D, 12:12 regime

The data in Table 4.1 and Figure 4.1 show that under these conditions cercariae emerged from their snail hosts almost entirely during

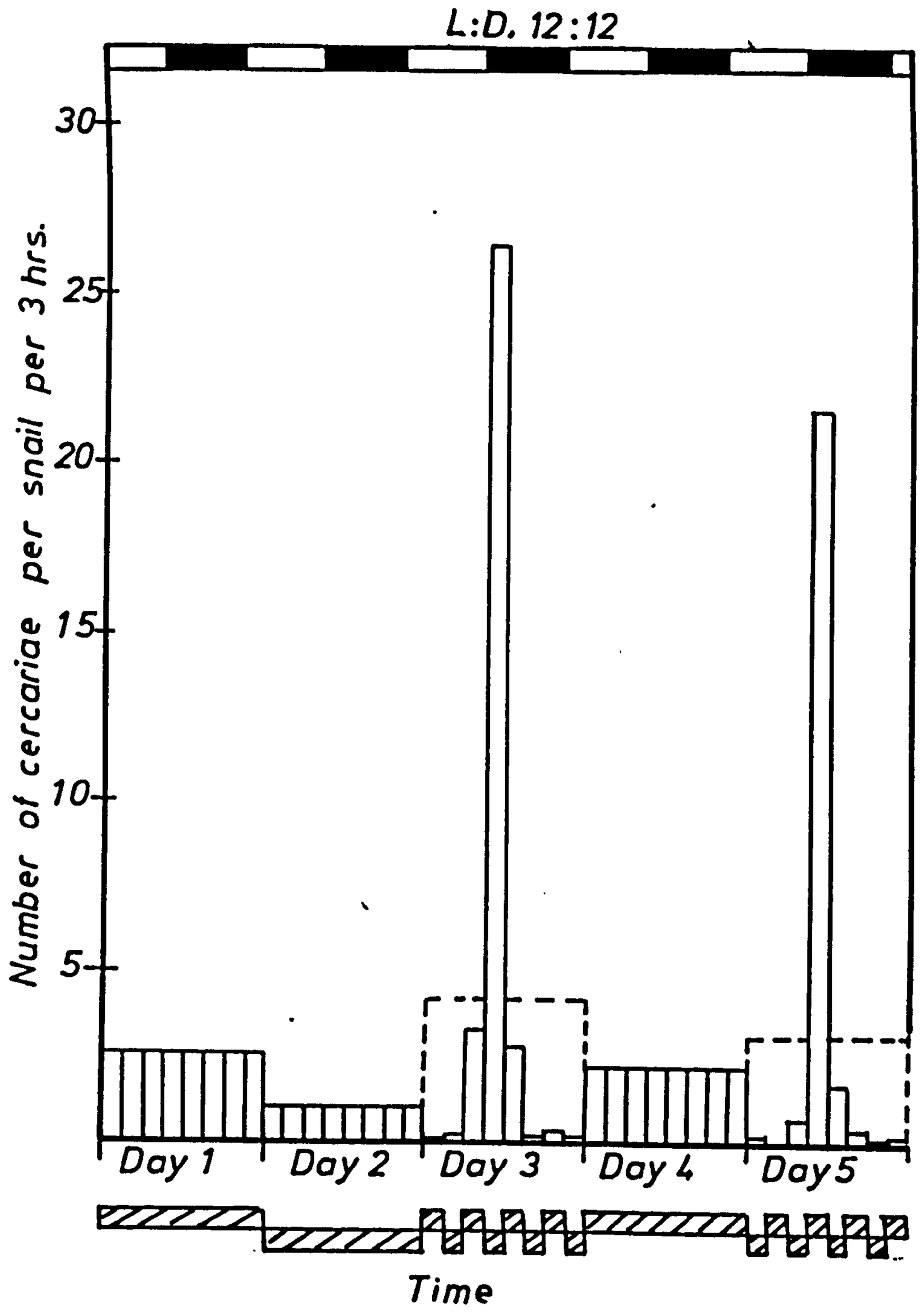
Table 4.1 Effects of L:D, 12:12 regime on the emergence of T. patialense, cercariae from the snail host M. tuberculata. (Dark began at 19.30hrs)

	Time	Number of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total no. of cercariae per 11 snails	Total no. of cercariae per snail	Total no. of cercariae per snail per 3 hours
Day 1	10.30-10.30	24	30	13	4	18	6	27	51	15	18	19	226	20.54	2.56
Day 2	10.30-10.30	17	10	7	15	0	1	8	1	17	10	0	86	7.81	0.97
Day 3	10.30-13.30	0	0	0	1	0	0	0	0	0	0	0	1		0.09
	13.30-16.30	0	0	0	0	0	0	0	0	1	1	0	2		0.18
	16.30-19.30	0	0	2	0	14	0	0	1	0	6	13	36		3.27
	19.30-22.30	35	5	13	32	24	14	36	38	28	23	43	290	33.36	26.36
	22.30-01.30	1	13	0	8	2	1	0	3	1	1	1	31		2.82
	01.30-04.30	0	1	0	0	1	0	0	0	0	0	0	2		0.18
	04.30-07.30	0	0	0	1	0	0	0	0	2	0	0	3		0.27
	07.30-10.30	0	1	1	0	0	0	0	0	0	0	0	2		0.18
Day 4	10.30-10.30	15	20	13	37	5	1	31	29	21	17	4	193	17.54	2.19
Day 5	10.30-13.30	0	0	0	1	0	0	0	0	0	0	1	2		0.18
	13.30-16.30	0	0	0	0	0	0	0	0	0	0	0	0		0
	16.30-19.30	1	0	1	0	2	0	0	0	0	3	1	8		0.73
	19.30-22.30	7	15	14	43	22	7	33	20	20	20	35	236	24.73	21.45
	22.30-01.30	0	1	0	5	3	2	0	5	2	1	0	19		1.73
	01.30-04.30	0	0	0	1	1	0	0	1	0	1	0	4		0.36
	04.30-07.30	0	0	0	1	0	0	0	0	0	0	0	1		0.09
	07.30-10.30	0	0	1	0	0	0	0	1	0	0	0	2		0.18

Figure 4.1 Effect of light L:D,12:12 regime on emergence of T. patialense cercariae from the snail host M. tuberculata. (Dark began at 19.30 hrs.)

The hatched zones below the X axis indicate the cercarial sampling periods.

Broken line represents the mean of three hours intervals.


L:D. 12:12
30
25
20
15
10
5
Number of cercariae per snail per 3 hrs.
Day 1
Day 2
Day 3
Day 4
Day 5
Time

the dark period with only very few cercariae emerging during each light period of the cycle. Combining the results from day 3 and day 5 of this experiment when three hourly cercarial counts were carried out, approximately 92% of the cercariae emerged during the periods of darkness and only 8% during the light.

The detailed three hourly counts on days 3 and 5 revealed that the 11 snails shed the great majority of their 24 hour total of cercariae within the first three hours of the dark period. On day 3 79% of the 24 hour total emerged during those three hours; on day 5 the same period revealed 87% of the day's total production. This concentration of the output into the period immediately after the initiation of the dark period was displayed almost invariably by the individual snails in this experimental population. Of the 22 snail/days monitored by constant three hourly sampling in this experiment only in one (Snail II, day 3) did the first three hours after the beginning of darkness fail to show the highest three hourly cercarial output over a 24 hour period. Figure 4.2 summarizes the cercarial output data from days 3 and 5.

During the five days of experiment 1 the total daily output of cercariae by the 11 snails varied between 86 and 367. There was no indication, however, that the different frequencies of experimental disturbance between days 1, 2 and 4 on the one hand and 3 and 5 on the other were linked with corresponding changes in output.

The relationship between the snail wet weight and the number of cercariae emerging on days 1, 3 and 5 of this experiment is shown in Table 4.2 and Figure 4.3. Inspection of Figure 4.3 suggests that there is unlikely to be any positive correlation between host size and levels of output of cercariae in this experimental population. Table 4.3 and Figure 4.4, however, reveal that cercarial size (as measured by mean head width in μm) does change in a patterned way during the five days of host starvation and cercarial emergence which were analysed. Cercarial head width declined rapidly between the first day after feeding stopped (mean width 606.8 μm) and the four succeeding days (mean values between 552.4 μm and 564.7 μm).

Figure 4.2 Effect of light L:D, 12:12 regime on emergence of *T. patialense* cercariae from the snail host *M. tuberculata* The mean of two days, three hours sampling. (Dark began at 19.30 hrs)

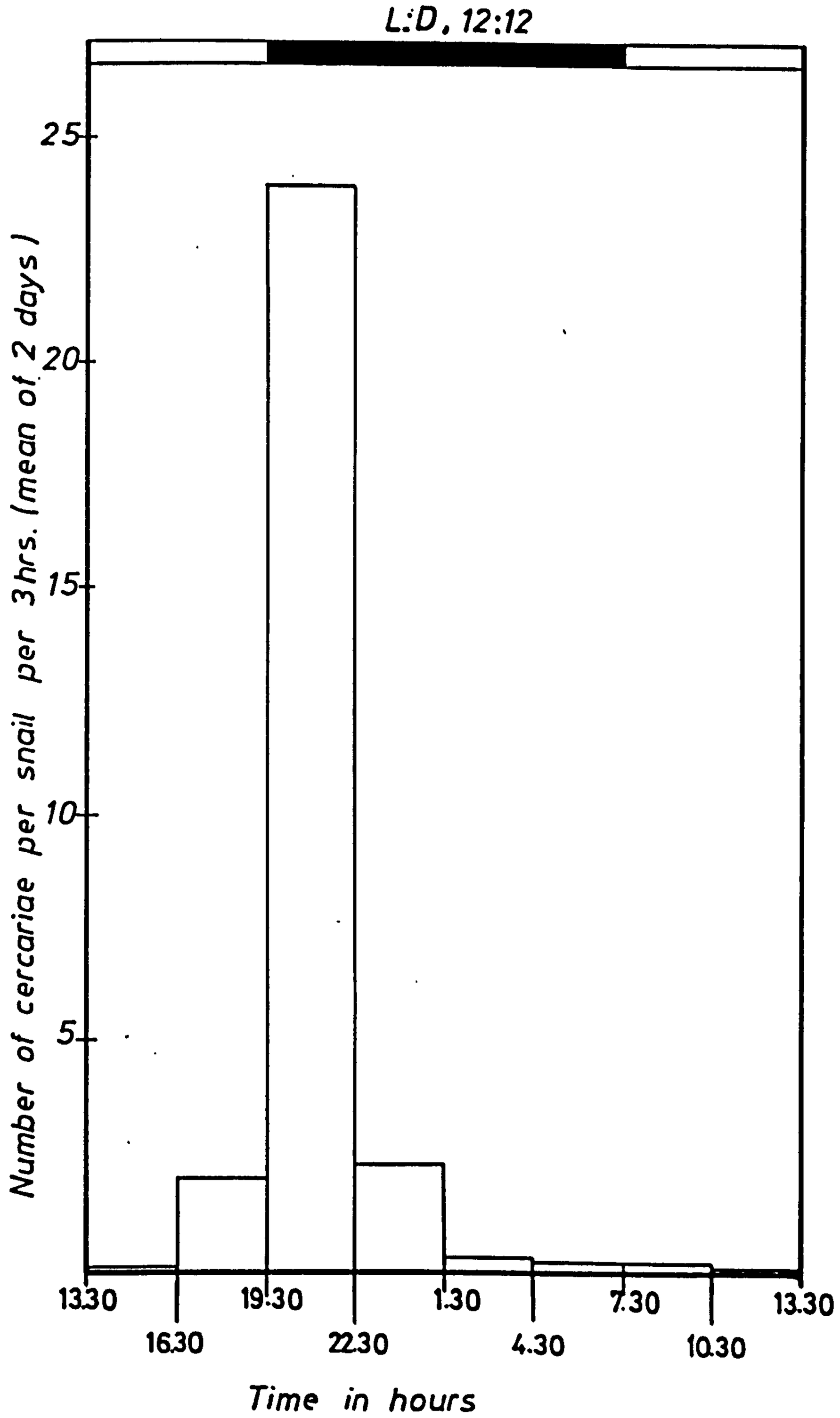

L:D, 12:12
25
20
15
10
5
Number of cercariae per snail per 3hrs. (mean of 2 days)
13.30
19.30
1.30
7.30
13.30
16.30
22.30
4.30
10.30
Time in hours

Table 4.2 The relationship between the weight of the snail and the number of cercariae emerging during days 1, 3 and 5 of the experiment

Snails number	Snail weight in grams	Number of cercariae emerging		
		Day 1	Day 3	Day 5
I	0.235	24	35	8
II	0.351	30	20	16
III	0.264	13	16	17
IV	0.505	4	42	48
V	0.194	18	41	28
VI	0.322	6	15	9
VII	0.236	27	36	33
VIII	0.362	51	42	27
IX	0.228	16	32	22
X	0.203	18	31	25
XI	0.207	19	57	37

Figure 4.3 The relationship between the weight of the snail and the number of cercariae emerging during days 1, 3 and 5 of the experiment

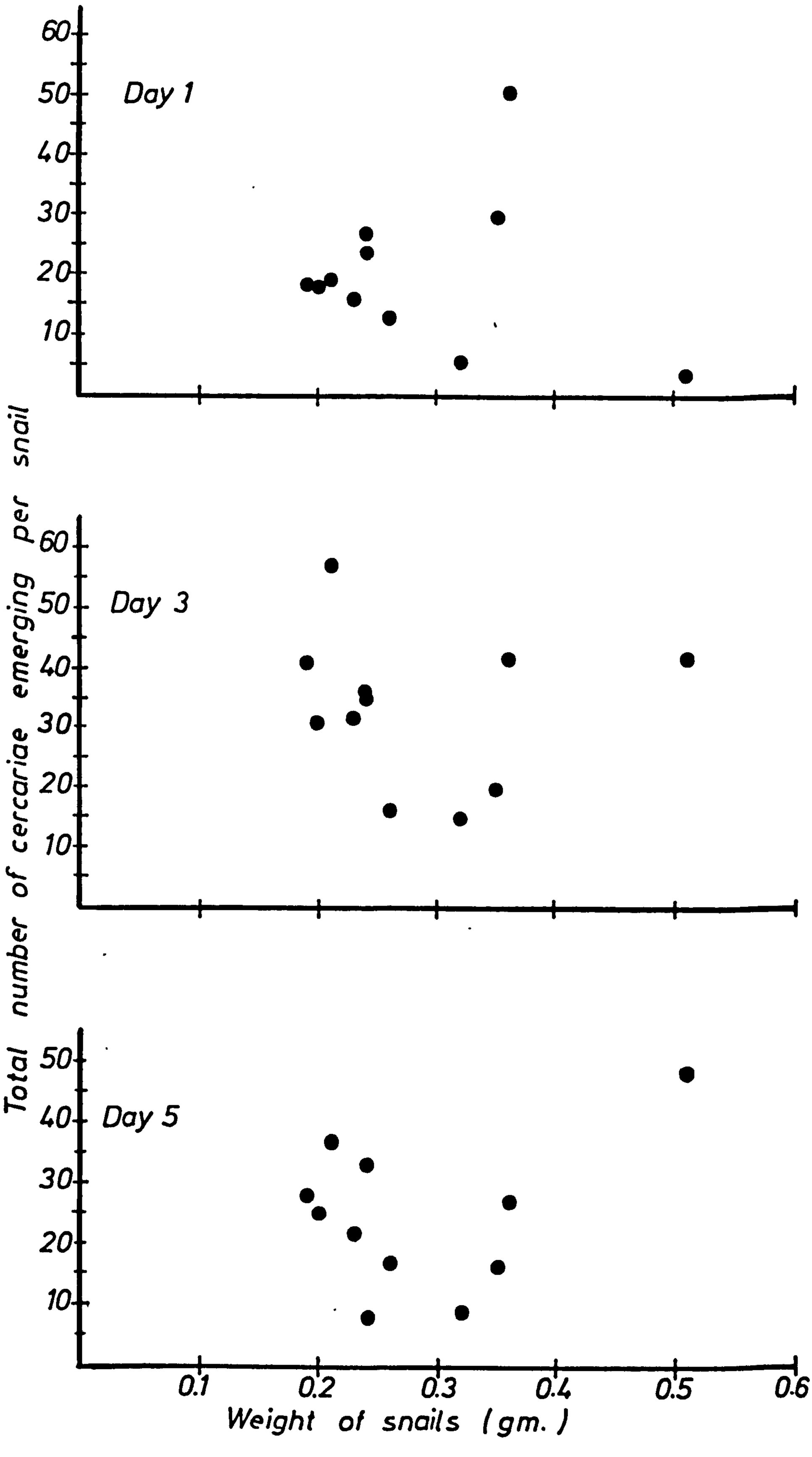
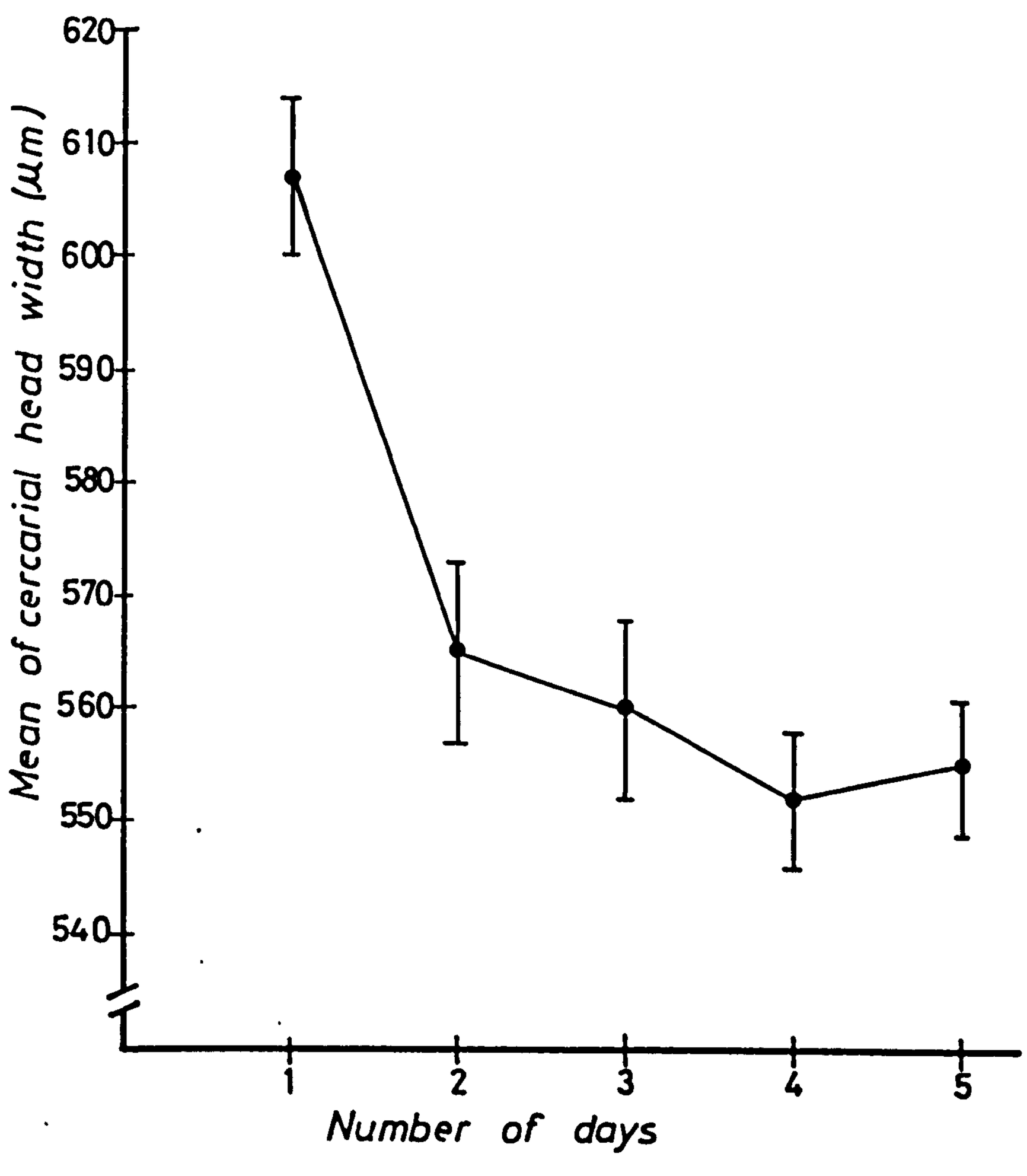

Day 1
Day 3
Day 5
60
50
40
30
20
10
Total number of cercariae emerging per snail
0.1
0.2
0.3
0.4
0.5
0.6
Weight of snails (gm.)

Table 4.3 The relationships between the weight of the snail and the head width of the cercariae, and the day of emergence

Snail number	Snail weight (g)	Mean cercariae headwidth in micron				
		Day 1	Day 2	Day 3	Day 4	Day 5
I	0.235	625.79	575.67	586.83	565.94	566.62
II	0.351	598.90	551.52	511.79	554.76	543.28
III	0.264	626.66	529.74	558.39	540.33	542.00
IV	0.505	608.22	568.98	595.96	558.77	595.57
V	0.194	587.81	0	573.59	547.40	555.25
VI	0.322	587.15	0	559.94	608.22	542.39
VII	0.236	620.68	592.72	577.12	556.62	586.44
VIII	0.362	646.48	608.22	588.11	543.96	551.32
IX	0.228	620.48	540.04	518.65	540.04	539.06
X	0.203	575.26	550.54	527.29	525.62	527.27
XI	0.207	577.32	0	565.35	534.65	559.17
Mean (±SE)		606.8 ± 6.7	564.7 ± 7.6	560.3 ± 8.3	552.4 ± 6.3	555.3 ± 6

Figure 4.4 The relationship between the mean cercarial head width and the day of emergence.

Vertical bars represent the standard error of the mean.


620
610
600
590
580
570
560
550
540
Mean of cercarial head width (μm)
1
2
3
4
5
Number of days

4.3.2 Experiment 2; L:D, 12:12 regime with sampling at short intervals

Experiment 1 showed that about 80% or more of the daily output of cercariae in a L:D,12:12 regime appear during the first three hours of darkness. In this experiment the 15 minute periods of observation gave more detailed information about the precise form of this major temporal pattern of cercarial emergence (see Table 4.4 and Figures 4.5a,b,c and 4.6).

The best representation of this pattern is that obtained by combining the results from days 3 and 5 over the four hour periods commencing one hour before the initiation of the dark period.

The cercarial output as a function of time follows a smooth unimodal curve, which is beginning to rise in the first 15 minute period after the initiation of the dark period and rises rapidly to a maximal value between 30 and 45 minutes into the dark period. Thereafter output rates decline more slowly, the overall curve being skewed to the right.

4.3.3 Experiment 3; L:D, 12:12 regime reversal

Reversal of illumination regime pattern resulted in all 11 snails exhibiting what in most respects constitutes a corresponding reversal of the pattern of shedding as shown in Table 4.5 and Figure 4.7. The consequence of regime reversal, however, is a complex pattern of cercarial output differing in more than one way from the previous temporal output pattern. Immediately after the time when reversal itself occurred (17.30 hrs day 10) a minor peak of cercarial output occurred. A much larger peak, however, coincided with the initiation of the 12 hour retarded dark period. Over the next 36 hours the cercarial output pattern seemed entirely analogous with the prereversal pattern, approximately 88% of the cercariae emerged during the period of darkness and 12% during the light.

4.3.4 Experiment 4; Regime change from L:D, 12:12 to LL (continuous light)

As shown in Table 4.6 and Figure 4.8 there was a considerable emergence of cercariae when the snails remained in continuous light and examination of the snails at two hour time intervals, indicated that there was a small scale emergence of cercariae corresponding to the time when the next 12 hours dark period would have begun had not a LL regime been instituted.

Table 4.4 The number of T. patialense cercariae emerging per 15 minutes sampling period under L:D, 12:12 regime.(Dark began at 19.30 hrs).

	Time	Number of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total no. of cercariae per 11 snails	Total no. of cercariae per snail	Total no. of cercariae per snail per 15 minutes
Day 1	10.30-10.30	0	3	0	2	1	0	4	14	2	8	3	37	3.36	0.04
Day 2	10.30-10.30	1	1	1	0	3	0	6	11	14	6	13	56	5.09	0.05
Day 3	10.30-18.30	0	0	0	0	0	0	0	0	0	0	0	0		0
	18.30-18.45	0	0	0	0	0	0	0	0	0	0	0	0		0
	18.45-19.00	0	0	0	0	0	0	0	0	0	0	0	0		0
	19.00-19.15	0	0.	0	0	0	0	0	0	0	0	0	0		0
	19.15-19.30	0	0	0	0	0	0	0	0	0	0	0	0		0
	19.30-19.45	0	0	0	0	0	0	0	0	0	0	0	0		0
	19.45-20.00	0	0	0	0	0	0	3	0	0	0	0	3		0.27
	20.00-20.15	0	0	0	0	0	0	4	2	7	1	0	14		1.27
	20.15-20.30	0	1	0	0	0	0	2	3	2	0	0	8	6.72	0.73
	20.30-20.45	0	0	0	0	0	0	3	2	2	0	1	8		0.73
	20.45-21.00	1	1	0	0	0	0	0	2	2	0	2	8		0.73
	21.00-21.15	0	0	0	4	0	0	1	3	4	2	1	15		1.36
	21.15-21.30	0	0	0	2	0	0	0	1	1	0	0	4		0.36
	21.30-21.45	0	0	0	1	0.	0	0	2	1	0	0	4		0.36
	21.45-22.00	0	0	0	0	0	0	0	1	1	0	0	2		0.18
	22.00-22.15	0	0	0	0	0	0	0	0	0	0	0	0		0
	22.15-22.30	0	0	0	1	0	0	0	1	0	0	0	2		0.18
	22.30-10.30	1	0	0	0	0	0	1.	2	1	1	0	6	0.55	0.01

Table 4.4 (continued)

	Time	Number of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total no. of cercariae per 11 snails	Total no. of cercariae per snail	Total no. of cercariae per snail per 15 minutes.
Day 4	10.30–10.30	1	0	0	0	1	0	2	3	0	2	5	14	1.27	0.01
Day 5	10.30–18.30	0	0	0	0	0	0	0	0	0	1	0	1	0.09	0.003
	18.30–18.45	0	0	0	0	0	0	0	0	0	0	0	0		0
	18.45–19.00	0	0	0	0	0	0	0	0	0	0	0	0		0
	19.00–19.15	0	0	1	0	0	0	0	0	0	0	0	1		0.09
	19.15–19.30	0	0	0	0	0	0	0	0	0	0	0	0		0
	19.30–19.45	0	0	0	0	0	0	0	0	5	1	0	6		0.55
	19.45–20.00	0	0	0	0	0	0	3	1	3	2	0	9		0.82
	20.00–20.15	0	0	0	1	0	0	7	7	7	5	3	30	14.37	2.73
	20.15–20.30	2	0	0	4	0	0	1	2	6	10	6	31		2.82
	20.30–20.45	1	3	2	5	0	0	2	4	6	0	2	25		2.27
	20.45–21.00	0	1	1	3	0	0	0	3	6	1	3	18		1.64
	21.00–21.15	0	1	0	2	0	0	0	5	4	1	0	13		1.18
	21.15–21.30	0	1	0	1	0	0	2	2	1	0	0	7		0.64
	21.30–21.45	0	0	0	3	0	0	0	0	0	0	0	3		0.27
	21.45–22.00	0	1	0	4	0	0	0	1	0	0	0	6		0.55
	22.00–22.15	0	0	0	2	0	0	0	0	0	0	1	3		0.27
	22.15–22.30	0	0	0	1	0	0	0	0	1	0	0	2		0.18
	22.30–10.30	0	0	0	0	0	0	0	0	1	2	0	3	0.27	0.006

Figure 4.5 a) The number of T. patialense cercariae emerging per 15 minutes sampling over four hours period in day 3. (Dark began at 19.30 hrs.)

b) The number of T. patialense cercariae emerging per 15 minutes sampling over four hours period in day 5. (Dark began at 19.30 hrs.)

c) The number of T. patialense cercariae emerging per 15 minutes sampling over four hours period (mean of day 3 and day 5). (Dark began at 19.30 hrs.)

L : D, 12 : 12

Day 3
a

1.4
1.0
0.6
0.2

10.30 12.30 14.30 16.30 18.30 20.30 22.30 0.30 2.30 4.30 6.30 8.30 10.30

Day 5
b

2.8
2.4
2.0
1.6
1.2
0.8
0.4

10.30 12.30 14.30 16.30 18.30 20.30 22.30 0.30 2.30 4.30 6.30 8.30 10.30

Mean of day 3 and day 5
C

2.0
1.6
1.2
0.8
0.4

10.30 12.30 14.30 16.30 18.30 20.30 22.30 0.30 2.30 4.30 6.30 8.30 10.30

Number of cercariae per snail per 15 minutes

Time in hour

Figure 4.6 The number of T. patialense cercariae emerging per 24 hours in response to L:D, 12:12 regime. (Dark began at 19.30 hrs.)

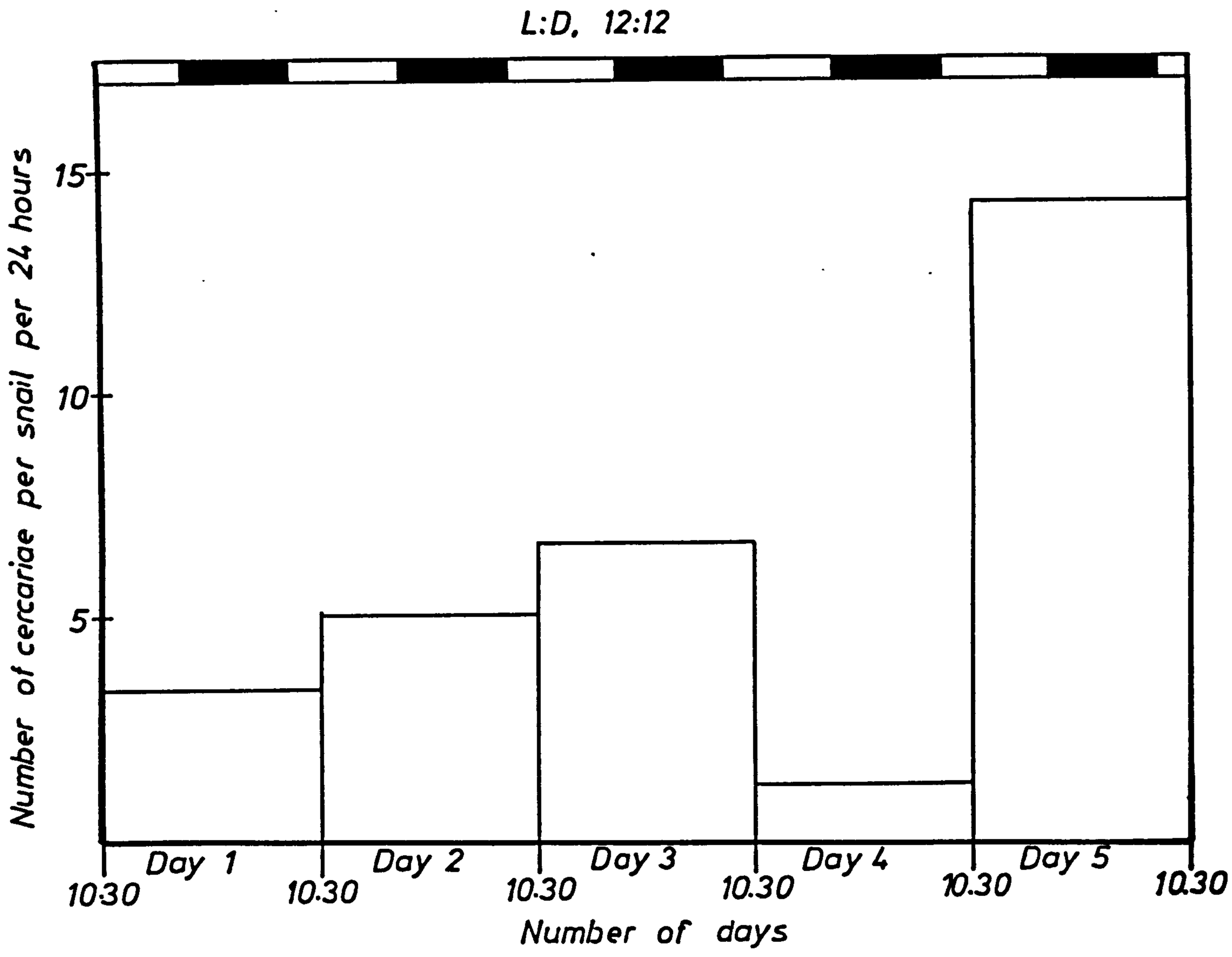

L:D, 12:12
Number of cercariae per snail per 24 hours
15
10
5
Day 1
Day 2
Day 3
Day 4
Day 5
10.30
10.30
10.30
10.30
10.30
10.30
Number of days

Table 4.5 The number of T. patialense cercariae emerging from the snail host M. tuberculata maintained under normal conditions for nine days and then under the conditions where the period of light and darkness were reversed for three days.(Dark began at 17.30 hrs).

	Time	Number of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total number of cercariae per 11 snails	Total number of cercariae per snail	Total number of cercariae per snail per 2 hrs
Day 1	10.30-10.30	23	12	1	7	9	7	7	1	11	2	3	83	7.55	0.63
Day 2	10.30-10.30	12	25	0	1	10	23	15	2	15	10	1	111	10.09	0.84
Day 3	10.30-10.30	15	16	2	12	5	17	10	9	16	7	0	119	10.82	0.90
Day 4	10.30-10.30	18	4	4	9	5	20	3	10	1	4	4	89	8.09	0.67
Day 5	10.30-10.30	20	6	4	4	1	9	0	7	7	2	4	84	7.64	0.64
Day 6	10.30-10.30	35	22	3	7	1	25	5	24	8	6	7	143	13	1.08
Day 7	10.30-10.30	23	3	5	3	1	11	0	1	6	4	2	59	5.36	0.45
Day 8	10.30-10.30	12	9	15	16	8	15	2	19	6	9	7	118	10.73	0.89
Day 9	10.30-10.30	11	4	15	7	2	8	0	22	4	5	2	80	7.27	0.61
Day 10	10.30-16.30	0	0	0	0	0	0	0	0	3	0	0	3	0.27	0.09
	16.30-18.30	1	0	0	0	0	1	1	0	1	0	0	4	0.36	
	18.30-20.30	1	2	2	0	0	0	0	0	1	3	0	9	0.82	
	20.30-22.30	0	0	2	0	0	4	0	0	3	0	0	9	0.82	
	22.30-0.30	0	1	0	0	1	0	1	0	0	2	0	5	0.45	
	0.30-2.30	0	0	1	2	0	1	0	0	0	1	0	5	0.45	
	2.30-4.30	1	0	2	0	0	0	0	0	0	1	2	6	0.55	
	4.30-6.30	35	1	38	28	0	0	0	22	2	9	0	135	12.27	
	6.30-8.30	3	7	4	7	0	13	0	7	2	1	2	46	4.18	
	8.30-10.30	0	0	1	4	2	9	0	1	0	0	0	17	1.55	

Table 4.5 (continued)

	Time	Number of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total number of cercariae per 11 snails	Total number of cercariae per snail	Total number of cercariae per snail per 2 hrs.
Day 11	10.30-12.30	1	0	1	0	0	0	1	0	0	0	1	4	0.36	
	12.30-14.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	14.30-16.30	0	0	0	0	0	4	0	0	1	1	1	7	0.64	
	16.30-18.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	18.30-20.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	20.30-22.30	0	0	0	0	0	0	0	0	0	1	0	1	0.09	
	22.30-00.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	00.30-2.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	2.30-4.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4.30-6.30	18	10	0	0	0	0	0	0	2	3	0	33	3	
	6.30-8.30	7	5	17	9	3	9	0	6	6	4	0	66	6	
	8.30-10.30	0	0	2	3	0	2	0	2	1	1	0	11	1	
Day 12	10.30-12.30	1	0	2	2	0	0	0	5	1	0	0	11	1	
	12.30-14.30	0	0	0	1	0	1	0	0	0	1	0	3	0.27	
	14.30-16.30	0	0	0	1	0	2	0	0	0	1	0	4	0.36	
	16.30-10.30	43	3	30	13	2	9	0	9	8	0	4	121	11	1.22

Figure 4.7 The number of *T. patialense* cercariae emerging from the snail host *M. tuberculata* maintained under normal condition for nine days and then under the conditions where the period of light and darkness were reversed for three days. (Dark began at 17.30 hrs.)

Single vertical bar represents average two-hourly cercarial output for each 24-hour period.

Broken line represents the mean of two hours interval.

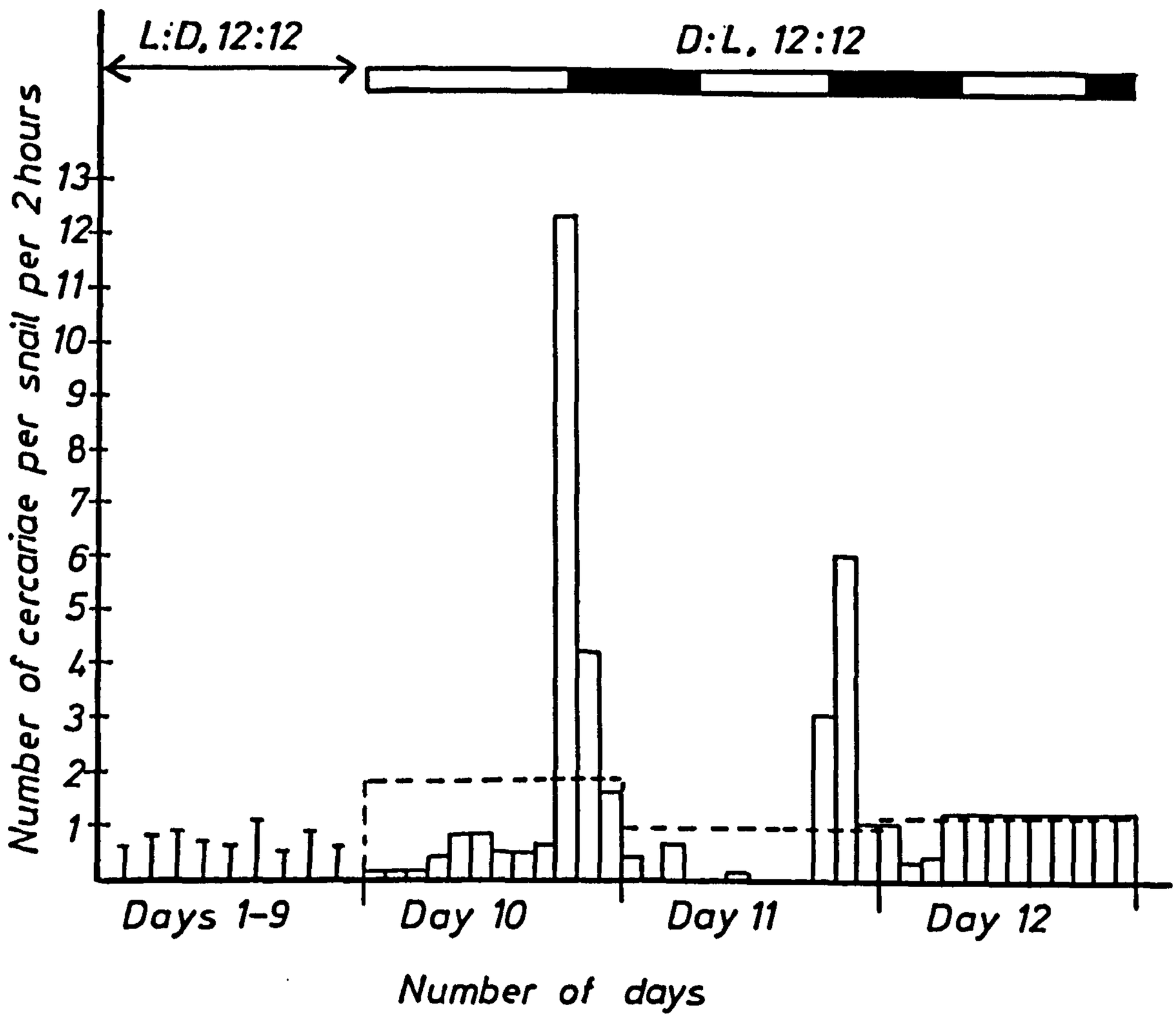

L:D, 12:12
D:L, 12:12
Number of cercariae per snail per 2 hours
13
12
11
10
9
8
7
6
5
4
3
2
1
Days 1-9
Day 10
Day 11
Day 12
Number of days

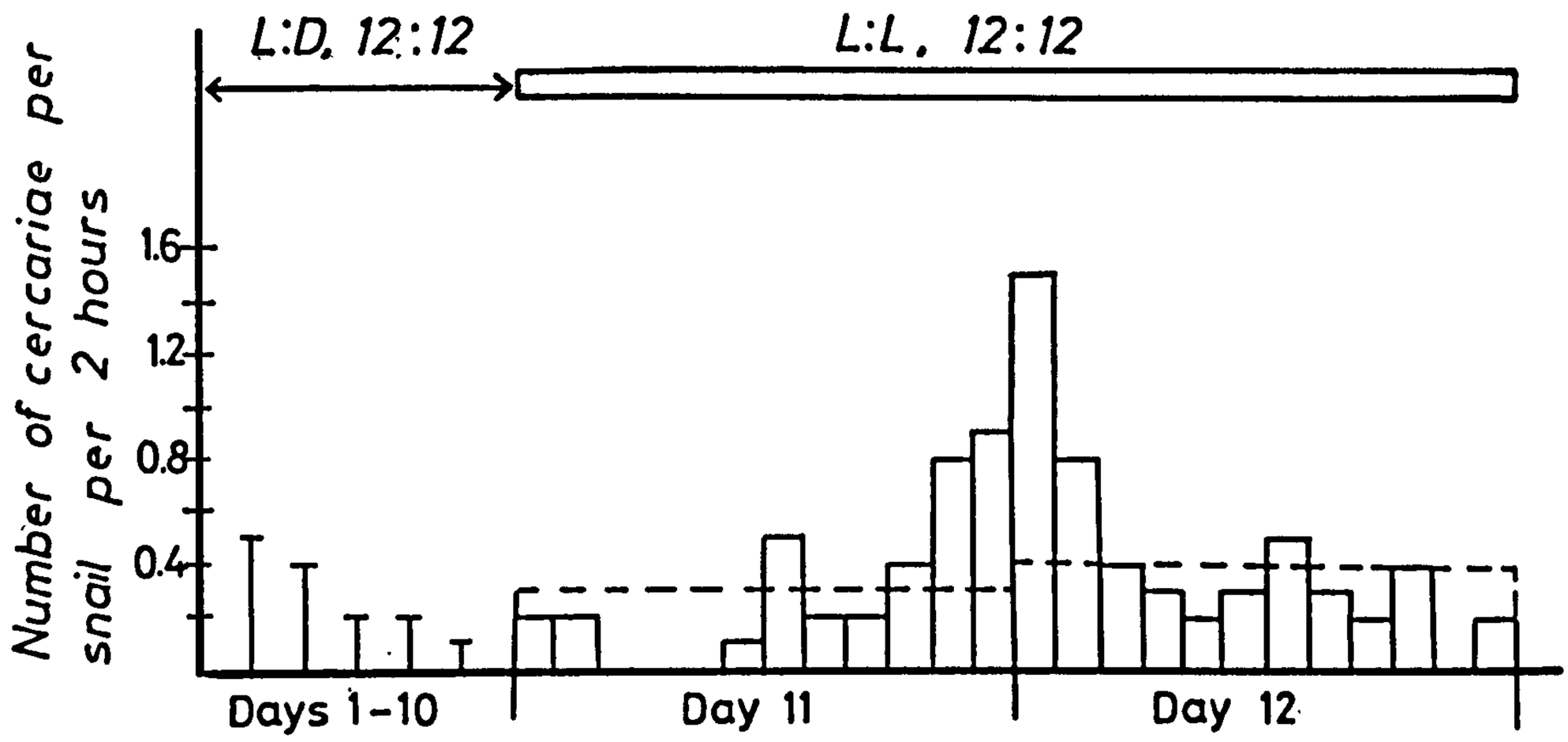
Table 4.6 The number of T. patialense cercariae emerging from the snail host M. tuberculata maintained under normal conditions L:D.12:12 for ten days and continuous light L:L,12:12 for 2 days. (Dark began at 22.00 hrs)

	Time	Number of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	Total number of cercariae per seventeen snails	Total number of cercariae per snail	Total number of cercariae per snail per 2 hours
Day 2	10-10 (48hr)	18	19	2	1	8	6	9	2	25	14	10	21	12	3	8	7	19	184	10.82	0.45
Day 4	10-10	3	10	4	3	15	4	7	3	8	33	3	5	10	9	1	6	22	146	8.58	0.36
Day 6	10-10	0	7	2	2	14	0	8	1	0	11	2	0	25	0	0	1	9	82	4.82	0.20
Day 8	10-10	0	21	2	3	10	0	4	0	0	17	0	0	19	1	0	3	0	80	4.70	0.19
Day10	10-10	0	9	1	4	3	0	11	0	0	17	0	0	10	0	0	3	1	59	3.47	0.14
Day11	10-12	0	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	0	4	0.24	
	12-14	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	3	0.18	
	14-16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	16-18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	18-20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	20-22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.12	
	22-0.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	8	0.47	
	0.0-2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	4	0.24	
	2-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	0.18	
	4-6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	6	0	7	0.41	
	6-8	2	0	0	0	2	0	0	0	0	4	0	0	2	0	0	3	0	13	0.76	
	8-10	1	0	0	1	2	0	0	0	0	1	0	0	10	0	0	1	0	16	0.94	
Day12	10-12	1	1	4	1	7	0	7	0	0	1	0	0	3	0	0	1	0	26	1.53	
	12-14	0	1	0	0	2	0	4	1	0	1	0	0	0	0	0	4	0	13	0.76	
	14-16	1	0	0	0	2	0	3	0	0	0	0	0	0	0	0	0	0	6	0.35	
	16-18	0	0	0	0	3	0	0	0	0	1	0	0	0	0	0	1	0	5	0.29	
	18-20	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	3	0.18	
	20-22	0	1	0	0	1	0	2	0	0	1	0	0	0	0	0	0	0	5	0.29	
	22-0.0	1	6	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	9	0.53	
	0.0-2	0	1	0	0	2	0	0	0	0	1	0	0	0	0	0	1	0	5	0.29	
	2-4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0	3	0.18	
	4-6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	6	0.35	
	6-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	8-10	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	4	0.24	

Figure 4.8 The number of T. patialense cercariae emerging from the host M. tuberculata maintained under normal condition L:D, 12:12 for 10 days and continuous light L:L, 12:12 for two days. (Dark began at 22.00 hrs.)

Single vertical bar represents average two-hourly cercarial output for each 48-hour period.

Broken line represents the mean of two hours interval.


L:D. 12:12
L:L. 12:12
Number of cercariae per snail per 2 hours
1.6
1.2
0.8
0.4
Days 1-10
Day 11
Day 12
Number of days

The rest of the 48 hours of detailed sampling revealed some cercarial release in almost every two hours sampling period; 20 out of 24 such periods exhibiting cercarial emergence. Despite this expansion of the emergence through each 24 hour period a marked peak was obvious, in a period corresponding to the time when the second L:D change would have been expected to occur.

Thus, in conditions of constant light, the cercarial emergence pattern retains (in a somewhat disturbed form) the approximately 24 hour rhythmicity of release shown in L:D, 12:12 conditions. This result suggests that some component of the snail/parasite system possesses an endogenous clock mechanism that can, at least partly, control the emergence pattern.

4.3.5 Experiment 5; Regime change from L:D, 12:12 to DD (continuous darkness)

The findings of Experiment 4 suggested that an examination of emergence in DD would be fruitful as behaviour in such conditions might be expected to provide information on the free running period a possible endogenous clock in the system.

The results of this experiment are shown in Table 4.7 and Figure 4.9. Cercariae continued to emerge in conditions of constant darkness and the two-hourly interval counts revealed that the basic cyclical pattern of emergence characterization of the L:D, 12:12 regime persists in a distorted form.

Once again, (cf Experiment 4) the trough periods of the cycle did not consist in general of no cercarial output at all. Instead almost all sampling periods (31 out of 36) showed some cercarial emergence. Despite this almost continuous "background" emergence the cyclical overall nature of the pattern was clear.

In an attempt to determine the apparent free running period of this behavioural cycle in DD conditions the data on emergence was recalculated as three-hour running means during days 9, 10 and 11 (Figure 4.10). This treatment produced a smoothed version of the output data from which two successive values of peak-peak time interval (i.e. cycle period) could be estimated. These were 28.2 and 28 hour. Thus the mean cycle period in free running D D conditions suggested by these results is about 28.1 hour.

Table 4.7 The number of T. patialense cercariae emerging from the host snail M. tuberculata maintained under normal conditions L:D,12:12 for 8 days and continuous darkness D:D,12:12 for 10 days (Dark began at 17.30).

	Time	No. of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total no. of cercariae per 11 snails	Total no. of cercariae per snail	Total no. of cercariae per snail per 2 hours
Day 1	10.30-10.30	8	7	8	9	1	20	0	7	15	12	1	88	8	10.67
Day 2	10.30-10.30	9	8	23	8	0	6	1	12	1	14	6	88	8	0.67
Day 3	10.30-10.30	7	9	14	7	1	6	7	4	15	8	7	85	7.73	0.64
Day 4	10.30-10.30	1	11	15	1	3	4	9	16	3	6	13	82	7.45	0.62
Day 5	10.30-10.30	0	11	6	4	12	14	4	3	10	13	4	81	7.36	0.61
Day 6	10.30-10.30	0	13	9	8	4	6	5	9	29	10	5	98	8.91	0.74
Day 7	10.30-10.30	0	5	14	11	16	17	9	1	9	10	10	102	9.27	0.77
Day 8	10.30-10.30	0	26	15	3	17	21	0	10	7	13	25	137	12.45	1.04
Day 9	10.30-16.30	0	0	0	0	0	1	0	0	0	0	0	1	0.09	0.03
	16.30-18.30	0	0	2	5	2	2	0	3	1	0	8	23	2.09	
	18.30-20.30	0	3	8	19	8	8	3	13	1	4	12	79	7.18	
	20.30-22.30	0	0	0	1	1	1	1	2	1	1	0	8	0.73	
	22.30-00.30	0	0	0	1	1	0	0	0	0	0	0	2	0.18	
	00.30-02.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	02.30-04.30	0	1	0	0	0	0	0	0	0	1	0	2	0.18	
	04.30-06.30	0	1	0	0	0	0	0	0	0	0	2	3	0.27	
	06.30-08.30	0	1	0	0	0	0	0	0	0	0	0	1	0.09	
	08.30-10.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
Day 10	10.30-12.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	12.30-14.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	14.30-16.30	0	0	0	0	0	0	0	0	0	0	0	0	0	
	16.30-18.30	0	1	0	0	0	1	0	0	0	0	1	3	0.27	

Table 4.7 (continued)

	Time	No. of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total no. of cercariae per 11 snails	Total no. of cercariae per snail	Total no. of cercariae per snail per 2 hours
	18.30–20.30	0	7	0	0	0	0	3	0	0	0	6	16	1.45	
	20.30–22.30	0	1	1	0	2	1	0	1	0	1	4	11	1.00	
	22.30–00.30	0	1	1	2	4	1	1	0	0	5	3	17	1.55	
	00.30–02.30	0	0	0	6	0	3	2	0	0	0	1	12	1.09	
	02.30–04.30	0	0	2	2	2	0	0	0	0	0	7	13	1.18	
	04.30–06.30	0	0	1	0	0	0	1	1	1	1	0	5	0.45	
	06.30–08.30	0	1	1	3	1	0	0	2	0	0	1	9	0.82	
	08.30–10.30	0	0	0	1	0	0	1	0	0	0	3	5	0.45	
Day 11	10.30–18.30	0	4	3	1	2	3	0	0	0	3	1	17	1.55	0.39
	18.30–20.30	0	0	0	0	1	0	0	0	0	0	0	1	0.09	
	20.30–22.30	0	2	0	2	1	0	1	0	0	0	0	6	0.55	
	22.30–00.30	0	3	1	2	3	0	0	3	0	1	1	14	1.27	
	00.30–02.30	0	2	0	2	2	2	0	0	0	2	0	10	0.91	
	02.30–04.30	0	0	0	0	5	3	2	3	0	3	8	24	2.18	
	04.30–06.30	0	1	0	3	5	1	1	0	1	0	3	15	1.36	
	06.30–08.30	0	0	0	0	2	1	0	1	0	2	1	7	0.64	
	08.30–10.30	0	2	0	4	0	0	2	0	0	0	4	12	1.09	
Day 15*	10.30–10.30	0	0	1	3	0	10	2	9	0	0	21	46	4.18	0.09
Day 16	10.30–10.30	0	21	2	4	13	7	6	6	0	10	12	83	7.55	0.63
Day 17	10.30–12.30	0	2	1	0	0	0	1	2	0	0	2	8	0.73	
	12.30–14.30	0	0	0	0	1	1	1	2	0	0	2	7	0.64	
	14.30–16.30	0	0	0	0	2	0	0	0	0	1	0	3	0.27	
	16.30–18.30	0	0	0	0	1	0	1	0	0	0	2	4	0.36	
	18.30–20.30	0	2	0	0	1	1	2	3	0	0	0	9	0.82	

Table 4.7 (continued)

	Time	No. of cercariae per individual snail I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total no. of cercariae per 11 snails	Total no. of cercariae per snail	Total no. of cercariae per snail per 2 hours
	20.30-22.30	0	2	0	0	0	1	0	0	0	2	0	5	0.45	
	22.30-00.30	0	1	0	1	0	2	1	0	0	1	1	7	0.64	
	00.30-02.30	0	0	0	0	1	0	0	0	0	0	2	3	0.27	
	02.30-04.30	0	2	0	0	3	0	0	0	0	1	0	6	0.55	
	04.30-06.30	0	2	0	0	0	0	5	0	0	1	1	9	0.82	
	06.30-08.30	0	0	0	3	0	2	1	0	0	0	1	7	0.64	
	08.30-10.30	0	0	0	1	1	2	0	0	0	0	1	5	0.45	
Day 18	10.30-10.30	0	0	0	4	2	0	2	2	0	7	3	20	1.82	0.15

* (keeping the snails with good food for 96 hours)

Figure 4.9 The number of T. patialense cercariae emerging from the snail host M. tuberculata maintained under normal condition of L:D, 12:12 for eight days and continuous darkness D:D, 12:12 for 10 days. (Dark began at 17.30 hrs).

Single vertical bar represents average two-hourly cercarial output for each 24-hour period.

Broken line represents the mean of two hours interval.

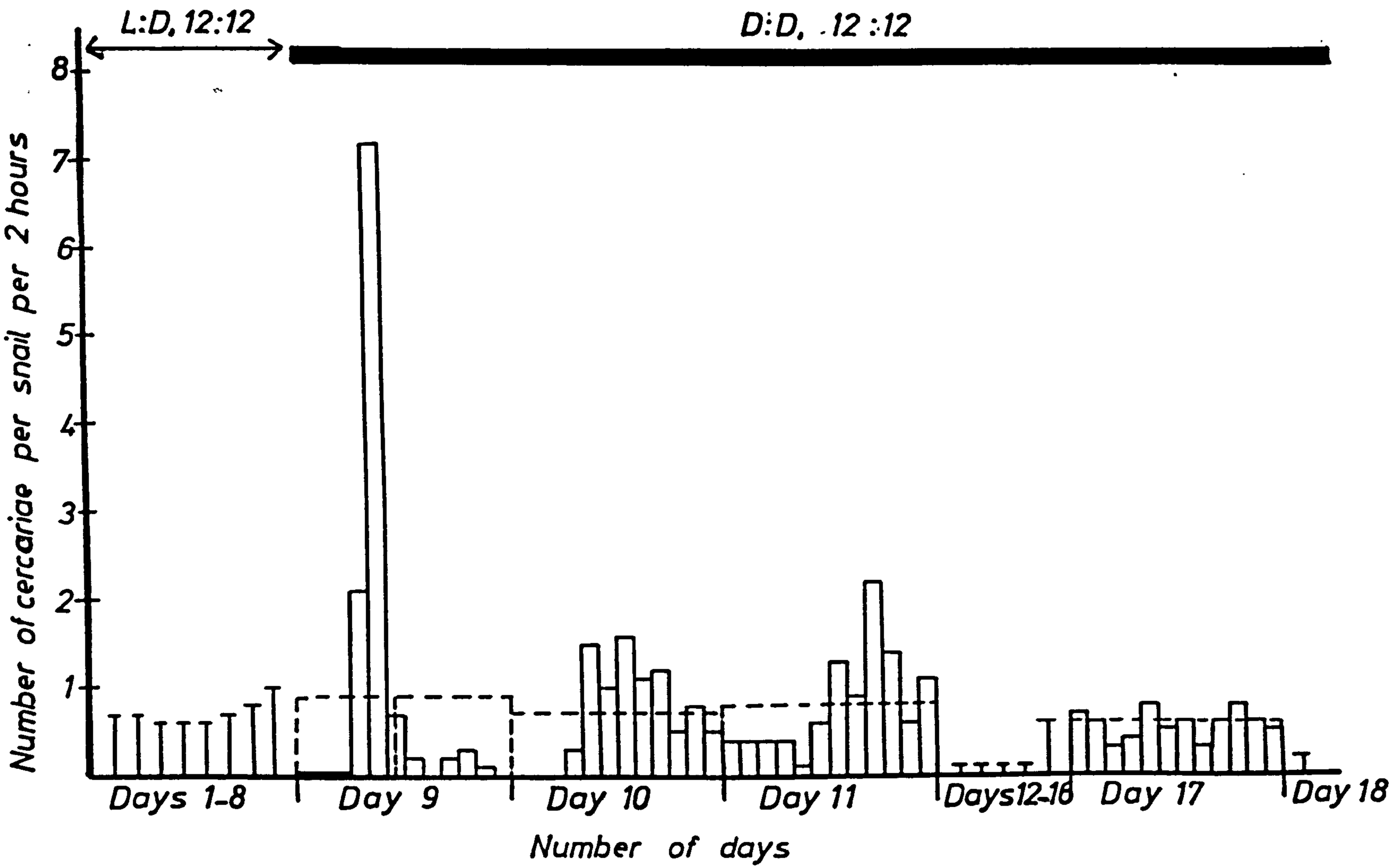
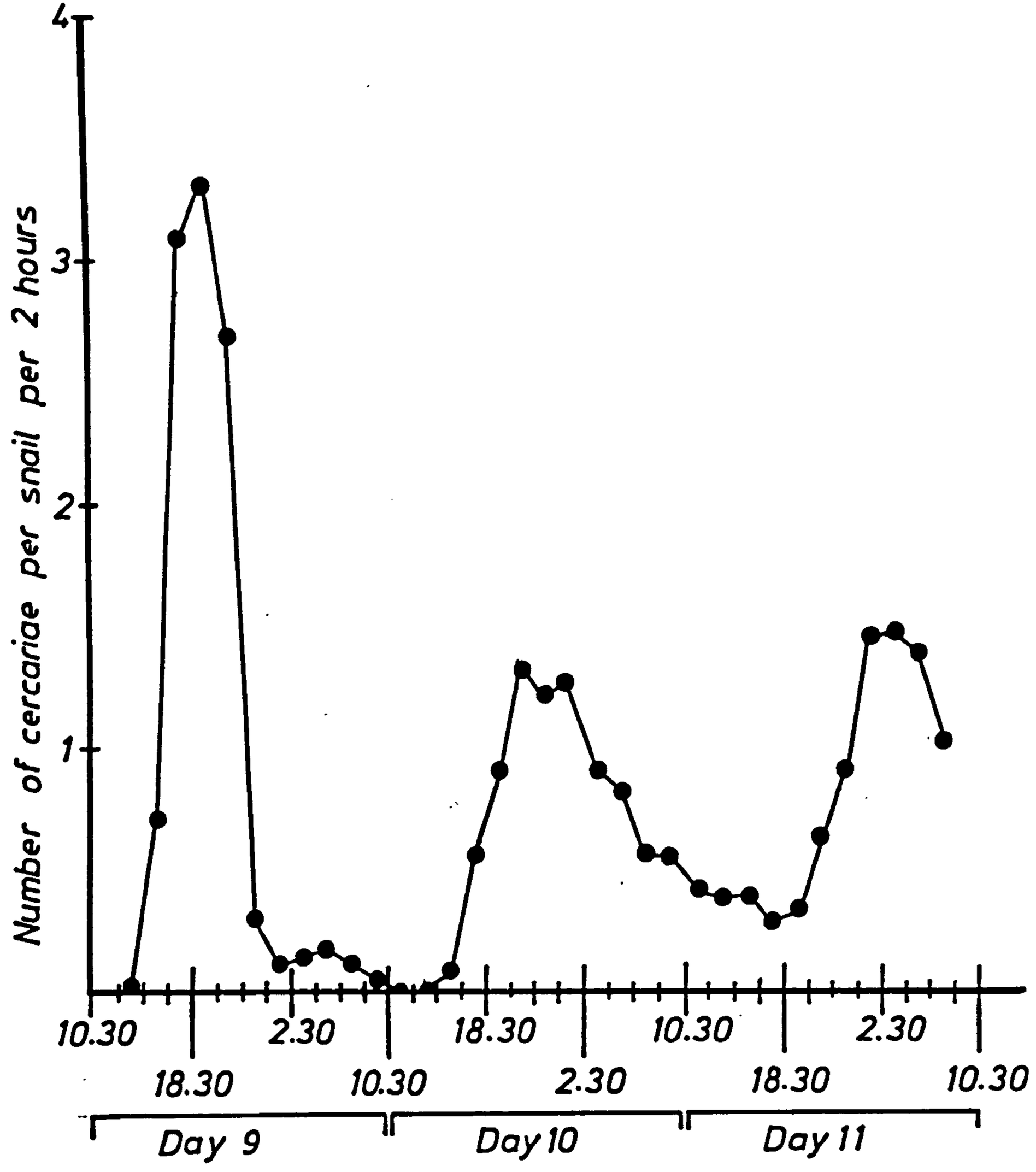

L:D, 12:12
D:D, 12:12
Number of cercariae per snail per 2 hours
8
7
6
5
4
3
2
1
Days 1-8
Day 9
Day 10
Day 11
Days 12-16
Day 17
Day 18
Number of days

Figure 4.10 The number of T. patialense cercariae emerging from the snail host M. tuberculata maintained under continuous darkness during days 9, 10 and 11, representing three hours running mean

The data points represent three hours running mean.


Number of cercariae per snail per 2 hours
4
3
2
1
10.30
2.30
18.30
10.30
2.30
18.30
10.30
2.30
18.30
10.30
Day 9
Day 10
Day 11

4.3.6 Experiment 6; Faecal production under L:D, 12:12 (12 hrs light: 12 hrs dark) regime

The data from two hourly monitoring of faecal output from 20 snails is presented in Table 4.8 and Figure 4.11. These results show that, not surprisingly, fed snails emit more faecal pellets than starved ones even though after five days of starvation both infected and uninfected starved snails were still producing some faecal pellets.

In all groups, whether fed or starved, infected with T. patialense or not, more faecal pellets were passed in the 12 hour duration of dark periods than in the preceding and corresponding 12 hour light periods. In both the fed populations there was some evidence of peaks in faecal output rate around the beginning and the end of the dark period.

4.4 Discussion

In this research programme, work on cercarial behaviour and locomotion within, or associated with, the first intermediate host of T. patialense has concentrated on migration within the snail (see Chapter 3) and the temporal patterning of such emergence. The results reported in this chapter have been concerned with the latter aspect of larval biology.

A wide variety of digenean species have been investigated in respect of temporal cercarial emergence patterns. Some results have come from uncontrolled experiments on natural snail populations and are relatively anecdotal, while others are from laboratory studies where many environmental variables can be kept under control.

The most conspicuous behavioural aspect of many examples of cercarial production is their periodicity with a cycle period of about 24 hours. The influence of photoperiod on this circadian periodicity has been studied in a number of investigations as summarized in Table 4.9 and Table 4.10 and a wide range of cercarial emergence patterns have been described. They can be listed under four headings;

(i) Irregular or constant output independent of photoperiod

Fasciola hepatica (Kendall & McCllough, 1951) is a typical example with emergence occuring under conditions of full illumination and in total darkness.

Table 4.8 The number of faecal pellets emerging from the snail host Melanoides tuberculata maintained under L:D, 12:12 regime

	Time	Group Ia Number of faecal pellets emerging per individual fed infected snail I	II	III	IV	V	Total no. of faecal pellets per 5 snails	Total no. of faecal pellets per snail	Total no. of cercariae per 5 snails	Group Ib No. of faecal pellets emerging per individual fed uninfected snail I	II	III	IV	V	Total no. of faecal pellets per 5 snails	Total no. of faecal pellets per snail
Day 11	10–12	1	0	35	18	71	125	25	2	88	6	2	12	18	126	25.2
	12–14	0	7	107	115	0	229	45.8		15	27	1	2	34	79	15.8
	14–16	0	43	7	87	2	139	27.8		4	0	0	0	61	65	13
	16–18	1	22	0	99	2	124	24.8		0	2	50	0	76	128	25.6
	18–20	0	51	0	55	0	106	21.2	2	14	4	21	0	73	112	22.5
	20–22	0	50	1	45	0	96	19.2	13	35	30	45	0	59	169	33.8
	22–0.0	5	54	15	73	39	186	37.2	23	45	36	14	0	71	166	33.2
	0.0–2	5	87	91	64	9	256	51.2	8	68	48	43	8	76	243	48.6
	2–4	29	31	79	35	0	174	34.8		46	6	23	0	42	117	23.4
	4–6	16	61	55	38	8	178	35.6		44	5	18	23	2	92	18.4
	6–8	34	73	36	0	72	215	43	1	48	13	78	16	10	165	33
	8–10	80	71	102	2	42	297	59.4		73	16	29	37	91	246	49.2
Day 12	10–12	5	83	37	0	33	158	31.6		78	7	2	60	51	197	39.6

Table 4. 8 (continued)

	Time	Group IIa No. of faecal pellets emerging per individual starved infected snail I	II	III	IV	V	Total no. of faecal pellets per 5 snails	Total no. of faecal pellets per snail	Total no. of cercariae per 5 snails	Group IIb No. of faecal pellets emerging per individual starved uninfected snail I	II	III	IV	V	Total no. of faecal pellets per 5 snails	Total no. of faecal pellets per snail
Day 11	10–12	1	0	2	1	0	4	0.8	4	0	0	0	0	0	0	0
	12–14	0	16	0	0	2	18	3.6		0	0	0	0	0	0	0
	14–16	0	26	2	0	2	30	6		0	0	0	0	4	4	0.8
	16–18	0	18	2	0	0	20	4	1	0	0	0	0	86	86	17.2
	18–20	0	27	0	0	0	27	5.6	1	0	0	0	0	82	82	16.4
	20–22	0	9	0	0	24	33	6.6	4	6	0	0	12	17	35	7
	22–0.0	49	11	0	0	33	83	18.6	34	4	0	1	0	0	5	1
	0.0–2	33	5	14	19	0	71	14.2	19	7	26	15	0	2	50	10
	2–4	13	10	25	15	0	63	12.6	1	0	24	33	4	9	70	14
	4–6	3	2	9	9	12	35	7	2	11	5	0	0	1	17	3.4
	6–8	9	0	18	0	0	27	5.4		20	19	0	1	1	41	8.2
	8–10	10	8	3	17	14	52	10.2	1	53	1	0	15	35	104	20.8
Day 12	10–12	34	2	2	4	0	42	8.4	2	0	0	5	4	1	10	2

Figure 4.11 The number of faecal pellets released from the snail host, M. tuberculata maintained under L:D, 12:12 regime

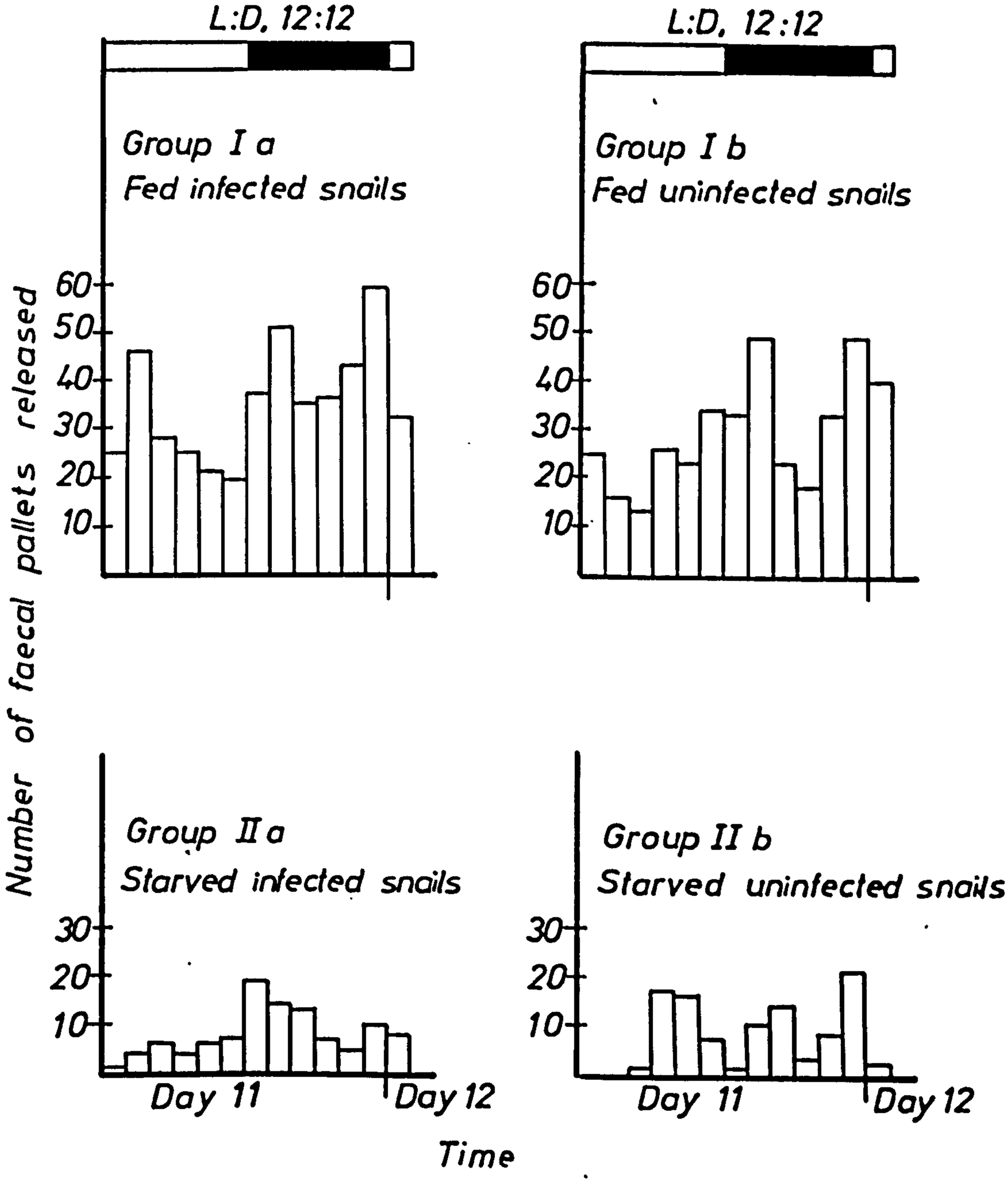

L:D, 12:12
Group I a
Fed infected snails
L:D, 12:12
Group I b
Fed uninfected snails
Number of faecal pallets released
60
50
40
30
20
10
Group II a
Starved infected snails
Group II b
Starved uninfected snails
30
20
10
Day 11
Day 12
Day 11
Day 12
Time

Table 4.9 A summary of a selection of digenean species exhibiting circadian rhythmicity of cercarial emergence under 12 hour cycles of alternate light and darkness

Parasite	Snail intermediate host	Peak of emergence Light/Crepuscular/ Dark		Reference
Cercaria elephantis	Planorbis trivolvis	*		
Cercaria emarginatae	Lymnaea emarginata angulata	*		Cort, 1922
Echinostome cercariae	Physa ancillaria parkeri	*		
Stylet cercariae	"	*		
Cercaria limbifera	Lymnaea palustris	*		
Cercaria "Z"	L. peregra	*		Rees, 1931
C. cambrensis I	L. truncatula		*	
Cercaria sp (closely related to) Cercaria brevifurca	Heliosomatrivolvis	*		
Diplostomum flexicandum	Stagnicola emarginata angulata	*		Giovanola, 1936
Cercaria sp of the subfamily Reniferinae	Physella gyrina	*		
Cercaria purpurae	Nucella lapillus	*		Rees, 1948
Schistosomatium douthitti	Lymnaea stagnalis		*	Olivier, 1950
	L. palustris			
Fasciola hepatica	Lymnaea truncatula	*	*	Kendall & McCllough, 1951
Plagiorchis vespertilionis parorchis	Lymnaea stagnalis		*	Macy, 1959
P. micracanthos	Stagnicola exilis		*	Wagenbach & Alldredge, 1974

Table 4.9 (continued)

Parasite	Snail intermediate host	Peak of Emergence Light/Crepuscular/ Dark	Reference
P. noblei	S. reflxa	*	Blankespoor, 1977
	Lymnaea stagnalis		
Cotylophoron cotylophorum	Indoplanorbis exutus	*	Verma, 1961
Ribeiroia marini	Biomphalaria glabrata	*	Théron, 1975
Schistosoma mansoni	Australorbis glabratus	*	Kuntz, 1947
"	"	*	Luttermoser, 1954
"	Biomphalaria salinarum		Pitchford, Meyling, Meyling & Dutoit, 1969
	Bulinus (Physopsis) sp	*	
"	Biomphalaria glabrata	*	Valle, Pellegrino & Alvarenga, 1971
"	"	*	Ash, 1972
"	"	*	Glaudel & Etges, 1973
"	B. pfeifferi	*	Nojima & Sato, 1978
"	B. glabrata	*	Nojima & Sato, 1982
Schistosoma haematobium	Bulinus (Physopsis) sp	*	Pitchford, Meyling, Meyling & Dutoit, 1969
	B. truncatus		
"	Bulinus globosus	*	Nojima & Sato, 1978
"	"	*	Nojima & Sato, 1982
S. bovis	Bulinus truncatus	*	
S. mattheei	B. (Physopsis) sp	*	Pitchford, et al., 1979
S. rodhaini	Biomphalaria salinarum	*	

Table 4.9 (continued)

Parasite	Snail intermediate host	Peak of Emergence Light/Crepuscular/ Dark		Reference
Schistosoma japonicum	Oncomelania formosana	*	*	Isobe, 1923
"	Japanese strain snail	*	*	Osaka, 1938
"	Oncomelania quadrasi	*	*	Bauman, Bennett Bennett & Ingalls, 1948
"	O. hupensis	*		Mao, Li & Wu, 1949
	O. nosophora	*		Gumble, Otori, Ritchie & Hunter, 1957
"	O. quadrasi	*		Nojima, Sato, Blas & Kamiya, 1980
Diplostomum spathaceum	Lymnaea stagnalis		*	Andreyuk, 1979

Table 4.10 Experiments on cercarial emergence in which the L:D illumination regime has been experimentally manipulated

Parasite	Snail Intermediate host	Experimental condition a	b	c	d	Reference
Cercaria limbifera	Lymnaea palustris	*				Rees, 1931
Cercaria "Z"	L. peregra	*				
C. cambrensis I	L. truncatula	*				
Cercaria sp (closely related to) Cercaria brevifurca	Heliosomatrivolvis			*		
Diplostomum flexicandum	Stagnicola emerginata angulata			*		Giovanola, 1936
Cercaria sp of the subfamily Reniferinae	Physella gyrina			*		
Schistosoma mansoni	Australorbis glabratus	*	*			Kuntz, 1947
S. mansoni	A. glabratus			*		Luttermoser, 1954
S. mansoni	Biomphalaria glabrata	*	*			Valle, Pellegrino & Alvarenga, 1971
S. mansoni	B. glabrata			*		Asch, 1972
S. mansoni	B. glabrata			*		Glaudel & Etges, 1973
S. mansoni	B. glabrata	*	*		*	Nojima & Sato, 1982
Schistosoma haematobium	Bulinus globosus	*	*		*	Nojima & Sato, 1982
Schistosomatium douthitti	Lymnaea stagnalis			*		Olivier, 1950
	L. palustris					
Cercaria purpurae	Nucella lapillus	*	*	*		Rees, 1948
Fasciola hepatica	Lymnaea truncatula	*	*			Kendall & McCllough, 1951
Plagiorchis vespertilionis parorchis	L. stagnalis			*		Macy, 1959
P. micracanthos	Stagnicola exilis	*	*	*		Wagenbach & Alldredge, 1974
Ribeiroia marini	Biomphalaria glabrata	*	*	*		Theron, 1975

Key to experimental conditions:

a. refers to constant darkness
b. refers to constant light
c. refers to reversal of dark/light cycle
d. refers to a shorter or longer cycle time

(ii) Cyclical cercarial output with diurnal peak

Several species of cercariae have been found to emerge mainly or only during daylight. They include Schistosoma mansoni, (Kuntz, 1947; Luttermoser, 1954; Asch, 1972; Glaudel & Etger, 1973); Cercaria limbifera and Cercaria "Z" (Rees, 1931) and Cercaria purpurae, (Rees, 1948).

(iii) Cyclical cercarial output with nocturnal peak

Other cercariae appear to emerge principally or solely during the dark portion of a photoperiod e.g. Schistosomatium douthitti (Olivier, 1950); Plagiorchis microcanthos (Wagenbach & Alldrege, 1974) and Ribeiroia marini (Théron, 1975).

(iv) Cyclical cercarial output with crepuscular peaks

E.g. Diplostomum spathaceum (Andreyuk, 1979).

(Refer to Table 4.9 for details.)

The responsiveness of cercarial emergence to photoperiod may be further complicated by parasite strain-specific, and host species-specific differences. Such complexity is revealed by the work that has been carried out on Schistosoma japonicum.

Apparently contradictory shedding results have been obtained from experiments done by different workers to determine the role played by environmental factors on the emergence of Schistosoma japonicum cercariae. Their findings suggested that parasite strain differences may influence the shedding of Schistosoma japonicum cercariae. Mao, Li & Wu (1949), using Oncomelania hupensis infected with the Chinese strain of S. japonicum stated that light plays an important role on diurnal emergence.

A similar result was obtained by Gumble, Otari, Ritchie & Hunter (1957) using Oncomelania nosophora infected with the Japanese strain of S. japonicum. They recovered significantly more cercariae from a number of infected snails under the influence of artificial light for periods of eight hours or less. They also noticed a suppression of emergence under total darkness. Contrary to their results, Osaka (1938) using naturally infected snails collected in Japan, concluded that light had no effect on emergence. Likewise, Isobe (1923), using naturally infected O. formosana, detected no influence of light on cercarial emergence; in fact, he reported that darkness was more favourable.

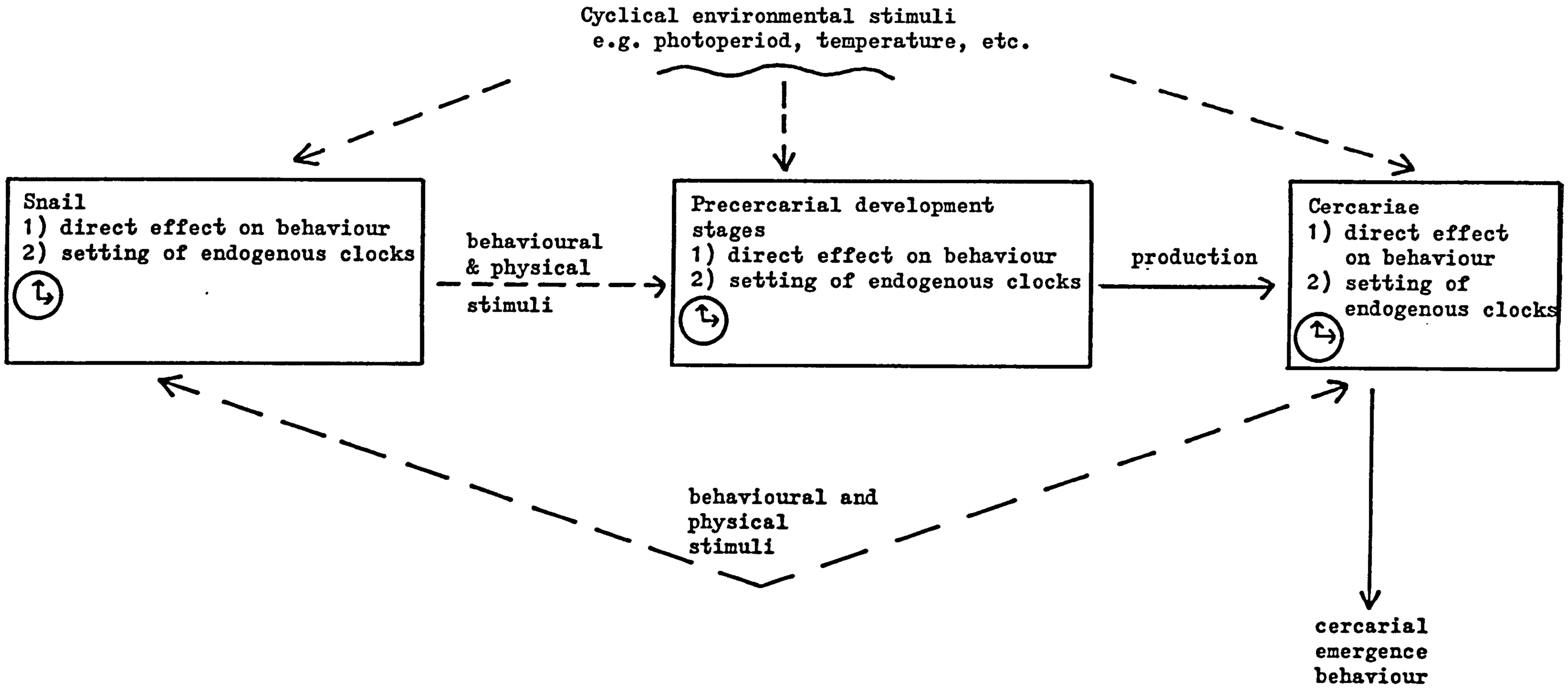
Although Bauman, Bennet & Ingalls (1948) concluded that neither light nor darkness is a critical factor in the escape of cercariae (Philippine strain) from O. quadrasi, they did note a nocturnal periodicity. In contrast, Nojima, Santor, Blas & Kamiya (1980) reported that under laboratory conditions the release of S. japonicum cercariae from Oncomelania quadrasi snails showed two patterns of emergence. Initially, non periodic emergence occurred immediately after submerging the snails in water and was followed by a periodic, diurnal emergence.

From their findings it appears, then, that emergence of cercariae of the several geographical strains of S. japonicum may not be stimulated equally by light-dark cycles.

The specific results presented in this chapter confirm and extend the earlier findings of Rao & Ganapati (1967) and Whitfield (1979) which showed that the cercariae of T. patialense emerge almost exclusively during the dark phases of light-dark cycles. It is known (Sim, 1972; Bundy, 1979) that during the life cycle of T. patialense, the miracidium penetrates the muscular foot of the snail host and develops into a sporocyst. The sporocyst undergoes asexual reproduction giving rise to a number of rediae. The rediae migrate to the digestive gland and probably after several generations of daughter redial production, give rise to furcocercous cercariae which develop freely in the haemocoelomic spaces of the digestive gland of the snail before emergence. This complex, multi-compartmental development makes analysis of the mechanisms that result in a particular temporal pattern of cercarial emergence very difficult. Figure 4.12 attempts, very diagrammatically, to illustrate these complexities.

Theoretically, the behaviour of snail hosts, precercarial larval developmental stages and cercariae themselves could be directly influenced by cyclical or discontinuous environmental stimuli. Equally, behaviour in each of the three cases could be altered by cyclical environmental clues (such as photoperiod phase) altering the "setting" of endogenous clocks in hosts or parasites by a "Zietgeber" effect (see Saunder, 1977). In addition, changes in snail behaviour and physiology will directly influence the activities of the developing parasites living within the snails. In a final layer of complication, the behaviour

Figure 4.12 Possible interactions that influence the pattern of cercarial emergence from the snail first intermediate host.


Cyclical environmental stimuli
e.g. photoperiod, temperature, etc.
Snail
1) direct effect on behaviour
2) setting of endogenous clocks
behavioural
& physical
stimuli
Precercarial development
stages
1) direct effect on behaviour
2) setting of endogenous clocks
production
Cercariae
1) direct effect
on behaviour
2) setting of
endogenous clocks
behavioural and
physical
stimuli
cercarial
emergence
behaviour

especially the asexual reproductive behaviour of sporocysts and rediae will influence the actual supply of cercariae for emergence.

Each of these components may be partially responsible for controlling the actual temporal patterning and magnitude of the nocturnal cercarial emergence of T. patialense, and some of the findings of the experiments carried out in this study give clues about the details of this control.

A distinct circadian pattern of emergence was obvious when the snails were subjected to L:D, 12:12 illumination regime. This circadian rhythm was characterized by a dark period peak of emergence which occurred within the first three hours of darkness. Approximately 92% of the cercariae emerged during the periods of darkness and 8% during the light (Table 4.1 and Figures 4.1 and 4.2.). From this evidence it can be seen that the emergence of T. patialense cercæriae is not continuously high throughout the whole dark period. It seems probable that this fact is related to a limited supply of mobile, mature cercariae that are capable of emerging, ultimately due to the finite level of reproduction of intramolluscan parasitic stages. It is assumed here that development of the cercariae goes on during the whole of any 24 hour period, and during the hours of light those which have developed to maturity will accumulate inside the snail to emerge in the next dark period. Thus a limitation on the hour to hour level of emergence is the rate of cercarial development.

With emergence monitoring carried out at short time intervals, cercarial output as a function of time was found to follow a smooth unimodal curve, which was beginning to rise in the first 15 minutes after the initiation of the dark period to reach a maximal value between 30 and 45 minutes into the dark period. This time lag is probably generated by differences in the time courses of the internal migrations of individual cercariae (see p.22 , Chapter 3). It appears that the majority of previously mature cercariae take about 30–45 minutes to move from the digestive gland to emergence sites. A few manage the migration faster and presumably others take longer, thus producing some of the skewed right hand tail of the emergence curve. During the dark period, however, new cercariae are presumably reaching maturity and will themselves begin migration. This latter population of larvae must constitute the principle reason for the skewed nature of the emergence curve.

An output curve with nocturnal periodicity has been reported by several researchers working on a variety of snail hosts and a variety of cercariae.

Olivier (1950), for instance, working on Schistosomatium douthitti cercariae found that snails subjected to 12 hrs of darkness from 6 pm to 6 am and 12 hrs of light from 6 am to 6 pm shed almost all of their cercariae in the six hour period between 4 pm and 10 pm with maximum emergence within the first hour of darkness. Similarly, Macy (1960) reported that cercariae of Plagiorchis vespertilionis parorchis were shed almost entirely at night and hourly monitoring showed that the five snails in the experiment shed their maximum number of cercariae during the hours preceding 9 pm, 10 pm, 10 pm, 11 pm and 1 am when they were maintained in dark room from 5 pm to 7 am.

The natural periodicity also reported by Wagenbach and Alldredge (1974) working on cercariae of Plagiorchis micracanthos from Lymnaea exilis showed marked nocturnal periodicity but the time of maximum emergence and the emergence pattern within the dark period varied greatly among snails. They found when sampling 10 snails hourly over one 12 hr dark period, two snails exhibited maximum emergence in the fifth hour of darkness, one in the eighth, six in the ninth and one in the twelfth. Blankespoor (1977) working on cercariae of Plagiorchis noblei from naturally infected lymnaeid snails and using hourly counts from three snails for 48 hours of natural light and darkness revealed that 72% of the total cercariae shed were between 8.30 pm and 5.30 am. Similarly, Theron (1975) demonstrated that Ribeiroia marini cercariae emerged from the snail host during the night according to a circadian rhythm and the number of cercariae shed by all snails remained relatively constant during the seven days period of the experiment, with variation from individual to individual. Hourly monitoring showed that shedding started immediately when the light went off with a maximum number attained during the first hour of darkness.

In the case of T. patialense, given that no cercariae are in the juxtarectal output sites in the light, (see Chapter 3), significant cercarial output within 15 minutes of the beginning of a dark period is an important finding. It means that cercariae

can move from the digestive gland to output sites in less than this time. Such movement could be triggered in a number of ways. The cercariae within the tissues of the snail possess rhabdomeric eyes (Whitfield, Anderson & Moloney, 1975; Bundy, 1979) and might be able to respond directly to a reduction in illumination level transmitted through the body of the snail. However Asch (1972), noticed normal Schistosoma mansoni cercarial emergence in relation to photoperiod from black pigmented snails Biomphalaria glabrata, although light transmission through them must be less than in the case of less pigmented hosts.

It is also possible that T. patialense cercarial emergence might be synchronized with snail physiological activity changes themselves controlled by endogenous clock mechanisms, or external cyclical clues.

When the light and dark periods were reversed (Experiment 3), the pattern of emergence immediately changed to follow the new photoperiod phase, with the maximal emergence occurring at the beginning of the new dark period (12.27 cercariae per snail). This shows the crucial nature of photoperiod in triggering emergence in that the great bulk of output follows the changed dark periods.

The immediate reversal of periodicity in the emergence of cercariae with the inversion of the light cycle has been reported previously for the nocturnally emerging Schistosomatium douthitti (Olivier, 1951); Plagiorchis vespertilionis parorchis (Macy, 1959); Plagiorchis micracanthos (Wagenbach & Alldredge, 1972) and in Ribeiroia marini (Theron, 1975). It was also demonstrated for the daytime emerging Cercaria brevifurca and cercariae species of the subfamily Reniferinae (Giovanola, 1936); Cercaria purpurae (Rees, 1948) and Schistosoma mansoni (Luttermoser, 1954; Asch, 1972 and Glaudel & Etges, 1973). In other species of cercariae a gradual adjustment and change in the pattern took place until the inversion of periodicity was established e.g. Diplostomum flexicandum (Giovanola, 1936).

The powerful influence of photoperiod in controlling T. patialense cercarial emergence raises the question of whether the photoperiod response is a direct one or mediated via an endogenous clock or clocks. The experiments 4 and 5 take us some way to answering this question.

In these experiments, the emergence patterns during continuous light and continuous dark have been investigated in order to establish whether the photoperiod is the prime determinant factor behind the circadian rhythmicity of the emergence of T. patialense cercariae, or whether there is endogenous factor operating with the exogenous one.

In a condition of continuous light (Experiment 4), the cercarial emergence pattern was retained (in a somewhat disturbed form). This finding strongly suggests that emergence is at least partly dependent on endogenous factors. In continuous darkness (Experiment 5), cercariae continued to emerge and the basic cyclical pattern of emergence characterization of the L:D, 12:12 regime persists with a periodicity of approximately 28 hours rather than the 24 hours demonstrated in normal conditions. Thus, there is clear evidence from the present observations that cercarial output is at least partially an endogenously cyclical process and that it freely runs in continuous darkness in the absence of photoperiodic clues and other possible cyclical Zeitgebers. The persistence of these rhythms in the emergence of cercariae held in continuous light and continuous darkness is clearly strong evidence for some form of endogenous control.

The present findings concerning the response of T. patialense cercarial emergence in continuous light or continuous darkness can be compared with those of other workers who have examined different digenean species. In some of these pieces of work similar responses have been found in that continuous darkness permitted emergence (Wagenbach & Alldredge, 1974; Theron, 1975). However, both sets of workers reported that continuous light suppressed cercarial emergence.

Suppression of the emission of the diurnal shedding Cercaria purpurae in the absence of any external time-cues has been reported by Rees (1948) when the snails were exposed to continuous darkness, while continuous light permitted cercariae to emerge continuously. In the latter conditions cercarial output is high at first, then falls, and finally rises to a new maximum. Rees explained that the fall in numbers may be due to the fact that the rate of development of the cercariae is not immediately increased to cope with the new stimulus situation.

Other workers have produced findings similar, in a general way, to those of this chapter. Innate emergence rhythms of this sort have been reported by Luttermoser (1955) for Schistosoma mansoni exposed to continuous light and dark, and by Kuntz (1947) who found a considerable liberation of Schistosoma mansoni from snail hosts in continuous darkness and also in continuous light conditions.

Similar results were obtained by Valle, Pellegrino & Alvarenga (1971) who found that under conditions of constant illumination or darkness, the rhythm of S. mansoni cercarial emergence continued to cycle with a period longer or shorter than 24 hours. Equally, Rees (1931) suggested that the periodicity of emergence in Cercaria limbifera, Cercaria "Z" and Cercaria cambrensis persists even in the absence of light but the number of cercariae produced is reduced.

Darkness duration in the day preceding sampling plays an important role in the amplitude and rhythmicity of the diurnal emergence of cercariae (Nojima & Sato, 1982). Suppression of Schistosoma mansoni and Schistosoma haematobium cercarial emergence was noted when the imposed dark period was very short (up to eight hours) in the preceding day. While a regular pattern of cercarial emergence was noticed when the dark period was 10-14 hours, patterns with two peaks in cercarial emergence were recognized in both species when the dark period was prolonged to 16 and 20 hours on the preceding day.

The observations of this study are compatible with the hypothesis that the snail-parasite system has an innate cercarial emergence rhythm with a period of about 28.1 hours rather than 24 hours, this period being revealed by exposing the snail to continuous darkness. In non-experimental conditions and experimental conditions with a light/dark cycle, it is assumed that the inherent frequency of the innate clock controlling the rhythm is reset (entrained) by the light/dark cycle.

The concept of endogenous (innate) control mechanisms in biological systems is based on a wide range of studies on organisms of all types (Saunders, 1977). The generalization can usually be made that the temporal control of biological functions is achieved by a balance between exogenous and endogenous factors. In few, if

any, systems does the latter operate alone.

Most "biological clocks" consist of an endogenous oscillator of unspecified location and morphology, whose rhythm is precisely entrained by some exogenous stimulus - the Zeitgeber - to run with a precise temporal period. It is evident that, in nature, organisms can rarely be under the kind of constant conditions where their rhythms freely run. If they were, they would lose any close relationship with the environment changes. What normally happens is that the inherent frequency of the endogenous clock controlling the rhythm is reset by the environment. The cockroach, for example, has an activity cycle which runs with a period of about 24.5 hours in constant darkness, so that it loses 0.5 hours per cycle relative to external solar time. When it is in a L:D, 12:12 regime, however, this drift is removed and its rhythm is reset the necessary half hour earlier each day by the arrival of experimental "sun set". (Brady, 1979).

This study has concentrated upon photoperiod as the crucial environmental determinant of emergence. No account has been taken of other environmental factors which have been considered to be important in other species by other workers. Temperature, for instance, can be expected to have threshold and graded effects on cercarial production.

With cercarial emergence, as with almost any biological process, over a large range of temperature one might expect to find the following types of phenomenon:

(i) upper and lower temperature thresholds for emergence;

(ii) an optimum temperature for emergence;

(iii) ranges of temperature over which emergence is dependent in a graded way on environmental temperature.

Table 4.11 summarizes previous work on the effect of temperature on emergence in different species revealing, among others, all the three phenomena described above.

Temperature is also of particular importance for the rate of larval development in the molluscan intermediate host. Nice & Wilson (1974) showed that exposure of Lymnaea truncatula to low temperature will reduce the overall rate of Fasciola hepatica development in proportion to the amount of time spent at low temperature.

Table 4.11 Effect of Temperature on the emergence of different species of cercariae

Parasite	Host snail species	Finding	Authors
Cercaria elephantis	Planorbis trivolvis	a	Cort, 1922
Cercaria cambrensis	Lymnaea truncatula	a	Rees, 1931
Schistosoma mansoni	Biomphalaria glabratus	b	Kuntz, 1947
Cercaria purpurae	Nucella lapillus	c	Rees, 1948
Schistosoma japonicum	Oncomelania hupensis	d,a	Mao, Li & Wu, 1949
Fasciola hepatica	Lymnaea truncatula	a	Kendall & McCllough, 1951
Schistosoma japonicum	Oncomelania nasophora	c	Gumble, Otori, Ritchie & Hunter, 1957
Austrobilharzia variglandis	Nassarius obsoletus	a	Sindemann, Rosenfield & Strom, 1957
Cotylophoron cotylophorum	Indoplanorbis exustus	e	Verma, 1961
Schistosoma mansoni	Biomphalaria salinarum		
S. haematobium	Bulinus (physopsis) sp		
S. haematobium	Bulinus (physopsis) sp		
	B. truncatus	a,b	Pitchford, Meyling, Meyling & Dutoit, 1969
S. bovis	B. truncatus		
S. rodhaini	Biomphalaria salinarum		
S. mattheei	Bulinus (physopsis) sp		
S. mansoni	Biomphalaria glabrata	b	Valle, Pellegrino & Alvarenga, 1973

a. represents level of cercarial output directly correlated with environmental temperature i.e. increase with temperature rise, decrease with temperature decline.

b. represents changes in water temperature in either direction causes changes in cercarial output.

c. represents upper or lower temperature threshold phenomenon.

d. represents output increased by temperature change.

e. represents data on optimal temperature for cercarial output.

The effect of salinity and pH of the water on cercarial emergence has been considered by several workers. Table 4.12 summarizes previous work on the effect of the salinity and pH of the water on the release of other species of cercariae from molluscan hosts.

Illumination cycles, temperature changes and alterations in ambient pH and salinity are all external environmental stimuli which have profound effects on cercarial emergence. Biological factors mediated through the snail host itself must also play a part in determining both levels of cercarial development and emergence. Perhaps the most important of these determinants are host size or age and host nutrient status.

The physical carrying capacity of the larval parasites' microenvironment is likely to affect both their development and asexual reproduction. Betterton (1979), also working on T. patialense, indicated that there is significant positive correlation between snail size and cercarial production rate. In the present work, the relationship between the number of cercariae emerging from each snail size category during days 1, 3 and 5 of Experiment 1, was studied. The results of this analysis showed that for snails in the wet weight range 0.203 - 0.505 g there appeared to be no significant degree of correlation between snail size and cercarial output. Anderson, Whitfield & Mills (1977) have produced similar results. This lack of correlation may be due to the small sample size and the limited size range of the hosts examined. It is also possible that the number of miracidia infecting each snail may affect the numbers of cercarial output. However, in the present study it is not known whether the snails were infected with single or multiple miracidia.

Intramolluscan digenean development might also be expected to be related to host nutritional status as these parasites are especially dependent on host tissues to sustain their growth. Host nutritional status might therefore be expected to affect cercarial development and output rates (Whitfield, 1981.) Indeed, Kendall (1949) noted that poor snail nutrition had a deleterious effect on cercarial development of Fasciola hepatica in the snail host.

Table 4.12 Effect of pH and Salinity of Water on the emergence of different species of cercariae

Parasite	Host snail species	pH	Salinity		Comments	Authors
			Fresh water	Marine water		
Cercaria purpurae	Nucella lapillus			*	b, 0.25N and 1.35N. Decreasing salinity to 0.375 reduces cercarial emergence. Increasing salinity to 1.375 inhibits cercarial emergence.	Rees, 1948
Austrobilharzia variglandis	Nassarius obsoletus			*	b, cercarial emergence at salinities between 15 and 25 parts per thousand, with marked reduction at higher and lower salinities.	Sindemann, Rosenfield & Strom, 1957
Schistosoma mansoni	Oncomelania quadrasi	C(6.4-7.6)	*		pH 7.6 is optimal, while at pH values below 7.2, cercariae did not emerge.	Bauman, Bennett & Ingalls, 1948
Schistosoma japonicum	Oncomelania hupensis	C(6.6-7.8)	*		Both these pH values suitable for cercarial emergence.	Mao, Li & Wu, 1949
Fasciola hepatica	Lymnaea truncatula	d(5.5-8.5)	*		pH value of water does not show any effect on cercarial emergence.	Kendall & McCllough, 1951
Cotylophoron cotylophorum	Indoplanorbis exustus	d(2-12)	*		No cercarial emergence at pH below 3 and above 11. Cercarial emergence appears to be mainly limited to a pH range of about 6.5-9.5.	Verma, 1961

a. represents output reduced by dilution of seawater (or lowered salinity levels)
b. represents upper or lower salinity threshold
c. represents pH optimum result
d. represents upper or lower pH threshold value

Eveland & Richie (1972) found that the well-nourished snails produce in general a denser cercarial suspension than those receiving an inadequate diet. Rich proteinoceous food (tropical fish food) was found to increase the rate of snail growth and to increase the number of Schistosoma mansoni cercariae emergence from Biomphalaria glabrata (Coles, 1973). Anderson et al., (1977) showed directly that the nutritional status of the snail hosts of T. patialense is an important determinant of larval parasite production. They found that the number of T. patialense cercariae produced per day per host dropped to zero approximately nine weeks after the hosts' food supply had ceased. They also found that the parasites could continue development and asexual reproduction once the food supply was resumed.

One secondary aspect of host nutrition which appears to be particularly important in relation to cercarial emergence in the Transversotrema patialense/Melanoides system is faecal output. The findings of Chapter 3 have demonstrated the prime importance of the rectal sinus for cercarial release. Although the detailed mechanisms of emergence into the outside world have not been investigated, there is every likelihood that the cercariae emerge into the external environment via the thin body wall over the rectal sinus or into the rectum itself or by both routes. Given this assumption it is reasonable to assume that localized muscular activity in the region of the rectum, especially peristaltic activity of the rectum itself could be implicated in the release mechanism. In this context the findings of this chapter on faecal output patterns deserve some close examination. More faeces were recovered in the dark than in the light period whether the snails were infected or not, fed or starved. Also there was some evidence of peaks in faecal output around the beginning and the end of the dark period in both the fed populations.

These patterns of faecal output mean that the emergence period of cercariae does correspond in time with a generally raised level of faecal output. Secondly, the peak at the beginning of the dark period coincides very precisely with the peak of normal cercarial emergence. Both these patterns of correlation increase the likelihood that there is some causal link between faecal output and cercarial emergence.

Any attempt to understand the ecological significance of the cercarial emergence pattern of Transversotrema patialense requires a detailed analysis of the temporal pattern of Melanoides behaviour and the behaviour of cercariae after their emergence.

Experiments on the freshwater prosobranch mollusc Melanoides tuberculata have demonstrated not only an endogenous rhythm in the locomotor activity of these animals, but one of a crepuscular nature (Beeston & Morgan, 1977). In their recent papers, (1979a,b) on the locomotor activity of Melanoides tuberculata these workers concluded that when the snails were maintained in naturally fluctuating day-night illumination conditions, the population showed a clearly defined cyclical pattern of activity. For most of the light period the majority of snails remained buried in the sand or gravel at the bottom of the aquarium, but emerged with the advent of darkness to graze on the bacterial and algal film on the surface of the substrata and lining the glass walls of the tank. This periodic migration has been readily observed in animals entrained to a L:D, 12:12 regime. Beeston & Morgan (1979a) recognised that besides the main period of activity at light off, an additional increase in activity was evident at the onset of the light period. They suggested initially that this behaviour is not a direct response to the light changes but may be controlled, at least in part, by an internal timing mechanism which is independent of the environment.

They noted that in constant light conditions and in constant reduced illumination conditions, the activity peaks drift relative to the times of the Zeitgeber, but retain the same temporal relationship to one another, suggesting that the free running period is the same for both peaks which is less 24 hrs in constant light and greater than 24 hrs in constant reduced illumination. In their subsequent research (1979b), however; they concluded that the two activity peaks of the crepuscular rhythm were in fact produced by two oscillators with no fixed relationship between the two.

There is thus a coincidence between the peak of T. patialense

cercarial emergence and the "dusk" crepuscular activity peak of their snail hosts. What ecological significance might this concurrence have?

T. patialense cercariae exist as free swimming larvae after their emergence from the snail. They have a complex locomotory pattern (Whitfield, Anderson & Bundy, 1977) which involve three different behavioural modes, namely active, tail-first swimming, passive dropping and resting periods. The maximum life span of cercariae maintained in tap water at a temperature of 24°C and 150 lux light intensity was found to be approximately 44 hours (Anderson & Whitfield, 1975). Survival characteristics, however, alone cannot adequately describe the total influence of increasing larval age on the biological functions of cercariae. The age-dependent changes in cercarial infectivity also must be taken into account. Cercariae progressively lose their capacity to infect fish and to develop to adulthood some time before they actually die. Whitfield et al,(1977) reported that infectivity decreased rapidly with time and larvae older than 22 hours failed to establish on the surface of the fish host in experimental conditions. In addition, cercarial predation by the fish host or other predators has been implicated in the reduction of cercariae populations (Anderson et al, 1977). It seems likely that the rate of infection of the final host will be dependent on the interaction between parasite/host and predator/prey population interactions. However, the rate of infection as well as the rate of predation both directly proportion to cercarial density (Anderson, Whitfield, Dobson & Keymer, 1978).

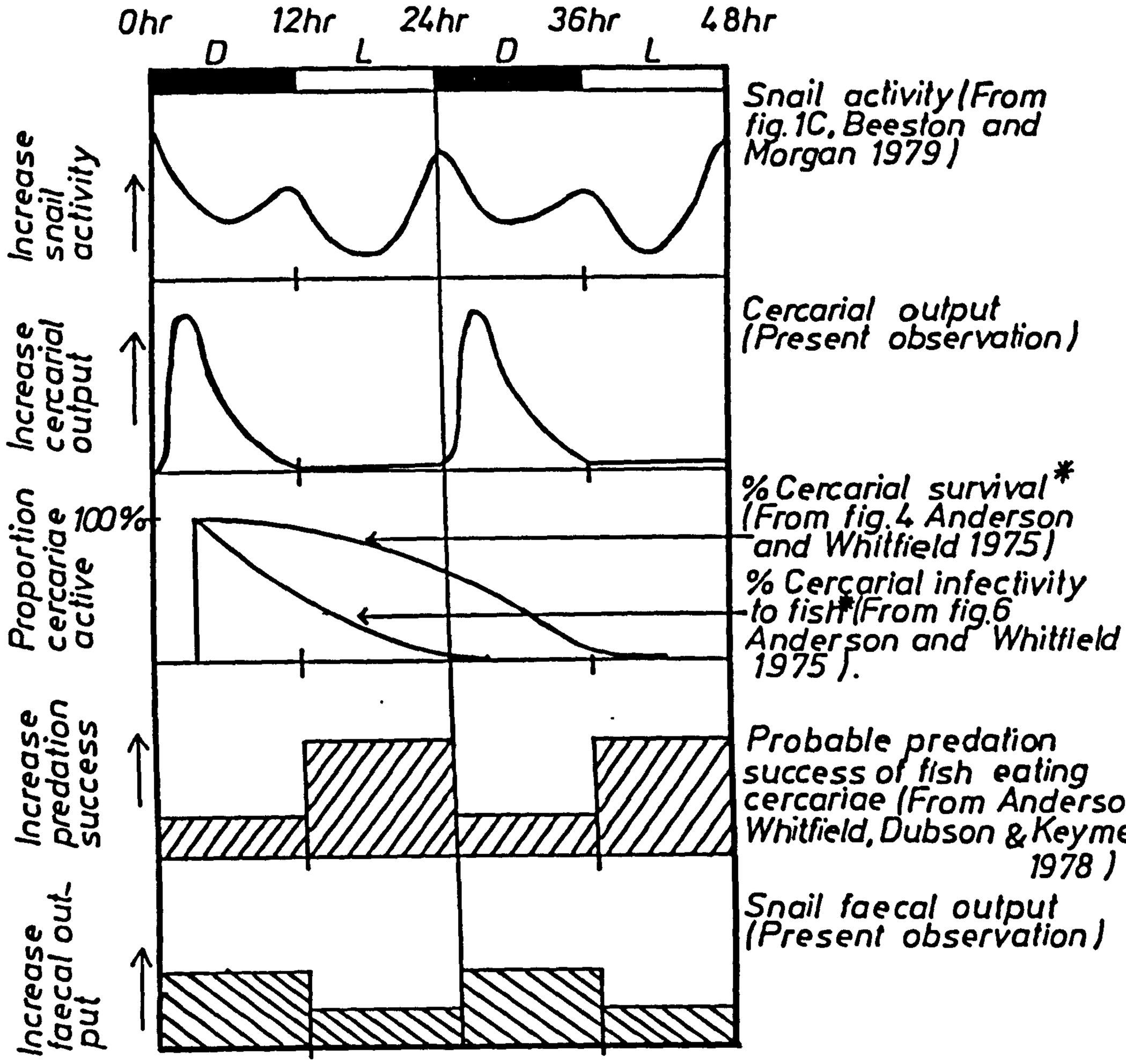
The aspects of Melanoides and cercarial behaviours described above can be combined with the cercarial emergence findings of this chapter to provide a general hypothesis to account for the selective values of the observed emergence pattern.

Figure 4.13 summarizes the time courses of the different behavioural modes and the implications of these time courses are listed below:

(i) The concentration of cercarial release into a relatively short segment of any 24 hr period.(especially in the first few hours of darkness) means that the chances of multiple cercarial

Figure 4.13 Schematic diagram of the time courses of aspects of Melanoides and cercarial behaviour.

* Cercarial survival and infectivity changes are illustrated only for the cercariae emerging on the first day and are considered to have all emerged at the time of peak emergence.


0hr
12hr
24hr
36hr
48hr
D
L
D
L
Increase snail activity
Snail activity (From fig. 1C, Beeston and Morgan 1979)
Increase cercarial output
Cercarial output (Present observation)
Proportion cercariae active
100%
% Cercarial survival*
(From fig. 4 Anderson and Whitfield 1975)
% Cercarial infectivity to fish* (From fig. 6 Anderson and Whitfield 1975).
Increase predation success
Probable predation success of fish eating cercariae (From Anderson, Whitfield, Dubson & Keymer, 1978)
Increase faecal output
Snail faecal output (Present observation)

infections of fish are enhanced. This fact will be of selective advantage in the case of T. patialense which is an obligatory cross fertilizing hermaphrodite (personal communication, Mr. Philip Espin, Kings College, Zoology Dept., London).
(ii) Cercarial release occurs at a time when snails are active and moving about in the aquatic habitat. This increases the likelihood of dispersion to fish hosts.
(iii) The time courses of cercarial infectivity and survival when considered in relation to the pattern of emergence mean that almost all infection encounters will take place in the dark when (a) fish might be moving less fast, or (b) fish cannot predate cercariae using visual clues.
(iv) Item (iii) above might explain why cercariae do not emerge in significant numbers at the time of the dawn peak of snails activity. Emergence at this time would expose cercariae to fish predation in the daylight hours.

The manner in which the periodicity in the emergence of cercariae might be an adaptation to the habits of the final host has been analysed in particular detail for schistosomes.

Cercariae of Schistosoma mattheei of sheep, Schistosoma bovis of cattle, sheep and goats, Schistosoma mansoni and Schistosoma haematobium of man, tend to emerge during the early part of the day (Pitchford, Meyling, Meyling & Dutoit, 1969). Schistosoma japonicum show a more complicated pattern of emergence with some cercariae being produced during day and night periods,which might suggest that this schistosome may have evolved as a parasite of man as well as of nocturnal mammals.(Bauman, Bennett & Ingalls, 1948). Schistosoma rodhaini and Schistosoma douthitti cercariae are shed at night when rodents serving as the final host are more active (Pitchford et al, 1969 and Olivier, 1950, respectively).

CHAPTER 5

An investigation of the development and mature organization of the nervous system of Transversotrema patialense cercariae using histochemical methods

5.1 Introduction

The previous two chapters have provided information concerning the migratory behaviour of cercariae of T. patialense within their snail intermediate hosts and the emergence behaviour of the same larvae.

Other aspects of larval T. patialense behaviour which have been investigated previously include the detailed nature of tail stem and furcal activity in tail-first cercarial swimming (Bundy, 1981a), the general behavioural repertoire of cercariae and their attachment behaviour in response to fish surfaces (Whitfield, Anderson & Moloney, 1975) and the age-dependent nature of the different components of cercarial activity (Whitfield, Anderson & Bundy, 1977). Incidental observations on the responsiveness of cercariae to both shadow stimuli and water turbulence have also been made (Anderson & Whitfield, 1975).

All these findings demonstrate, in an indirect manner, the undoubted complexity of the sensory and nervous systems of the larval developmental stages of T. patialense. To date, however, there have been no direct studies on the sensory equipment of larval T. patialense (apart from an analysis of cercarial arm process contact chemoreceptors by Whitfield et al., 1975) or the structure of their nervous systems. This relative lack of information is typical of the situation in respect of many other larval parasitic platyhelminths, although a range of different experimental techniques have been utilized in this area of investigation.

Early work utilized conventional light microscopical histological techniques (see, for instance, Zailer, 1914; Neuhans, 1952). More recently, several workers have found that the silver nitrate staining technique may be used for mapping tegumentary nerve endings in cercariae. The presence of sensory papillae on the body surface of cercariae has been described by various workers (for example, Seitner, 1951; Wagner, 1961; Lie, 1966; Chapman & Wilson, 1970; Mohandas, 1971; Richard, 1971; Short & Cartrett, 1973; Nuttman, 1975; Bayssade-Dufour, 1979; Grabda-Kazubska & Moczon, 1981; Niewiadomska & Moczon, 1982). This method, although excellent for studying the arrangement of sensillae, provides no information concerning

the non-tegumentary parts of the cercarial nervous system.

Recent electron microscopical studies have contributed greatly to the knowledge of the structure and function of the sensory components of nervous systems in parasitic platyhelminths. Scanning electron microscopical studies have proved particularly helpful in analysing patterns of surface receptors (see, for instance, Koie, 1971b; Short & Cartrett, 1973).

Transmission electron microscopical studies have revealed information concerning more central parts of the nervous system. The nervous systems of Fasciola hepatica and Schistosoma mansoni cercariae have, for instance, been studied by Dixon & Mercer (1965) and Nuttman (1975) respectively. Conventional transmission electron microscopy investigations, however, only have a restricted usefulness in the context of understanding large scale nervous system organisation. Partially in an attempt to overcome this limitation, a number of workers have utilised histochemical methodologies.

Histochemical methods based on the localisation of esterase activity have provided a fairly clear picture of the central nervous system structure of cercariae. These methods have been used by Lewert & Hopkins (1965), Fripp (1967), Bruckner & Voge (1974) and Nuttman (1975) working on Schistosoma mansoni; Jennings & Leflore (1972) working on Himasthla quissetensis and Zoogonus lasius; Leflore (1979) investigating Plagiorchis elegans; Leflore, Bass & Smith (1980) on Cloacitrema michiganensis; Grabda-Kazubska & Moczon (1981) on Haplometra cylindracea and Niewiadomska & Moczon (1982) on Diplostomum pseudospathaceum.

The present chapter records the results of experiments in which the nervous systems of intramolluscan developmental stages as well as cercariae of T. patialense were investigated with a variety of histochemical techniques. The methodologies utilised, sought to localise both acetylcholinesterase activity and biogenic amines, as both acetylcholine and the latter biogenic amines are good candidates for transmitter roles in parasitic platyhelminths.

5.2 Materials and Methods

The intramolluscan developing larval stages and mature cercariae were collected as described in Chapter 2 (2.3 and 4). Esterase activity in the nervous system of developing and mature cercariae

was demonstrated using the following methods.

Larval stages were placed in solid watch glass and fixed in 4% formalin. After fixation at room temperature for 20-30 minutes, the specimens were subjected to two five-minute washes in distilled water. After washing, they were treated by the three following histochemical procedures:

5.2.1 5-bromoindoxyl acetate method for non-specific esterases (after Holt & Withers, 1952). (5-BrI)

1.3 mg o-acetyl-5-bromoindoxyl (Sigma) was dissolved in 0.1 ml ethanol and after complete solution the following mixture was added:

0.05 M Tris buffer pH 7.0	2.0 ml
0.05 M potassium ferricyanide	1.0 ml
0.05 M potassium ferrocyanide	1.0 ml
0.1 M calcium chloride	1.0 ml
distilled water	5.0 ml

The specimens were incubated in this solution for 4-6 hours at room temperature. After completion of incubation, the specimens were washed twice in distilled water for five minutes. They were then mounted in Gurr water mounting medium on clean microscope slides and examined microscopically.

5.2.2 α-naphthyl acetate method of Nachlos & Sebgmain (as described by Pearse, 1960). (αNA)

10 mg α-naphthyl acetate (Sigma) was dissolved in 0.25 ml acetone and after complete solution 20 ml 0.1M-phosphate buffer (pH7.4) was added. Then 75 mg Fast Red Tr. salt was added as a coupler and the total mixture filtered directly onto the specimens.

The results of a series of preliminary tests on a series of diazonium salts indicated that the sharpest definition of staining was obtained when Fast Red Tr salt (Gurr) had been used. This salt was used routinely.

The optimum incubation time at room temperature was one to three hours, after which specimens were washed and mounted in water mounting medium, as described above.

5.2.3 Acetylthiocholine iodide method for cholinesterases (based on a modification of the Koelle & Friedenwald (1949)-Gomori (1952) method). (ATCI)

Newly emerged cercariae were fixed in Pearson's fixative (10 mls formaline, 1 ml 0.880 ammonia, 100 mls distilled water, 15 gms sucrose)

for 30 minutes at room temperature, washed twice in distilled water for five minutes, then incubated overnight at room temperature in a solution prepared by dissolving 100 mg of acetylthiocholine iodide (Sigma) in 130 mls of phosphate buffer (0.1M Na_2HPO4) pH6.0 and followed by subsequent addition with continuous stirring of 10 mls of 0.3M sodium citrate, 20 mls of 30 mM copper sulphate, 20 mls of distilled water and 20 mls of 15 mM potassium ferricyanide. After incubation, the cercariae were washed twice in distilled water for 10 minutes, then mounted and examined as described above.

In control experiments, to confirm the enzymic basis of the three histochemical reactions, the larvae were held at 90°C for five minutes prior to incubation, others were treated in the normal manner, but incubated in a media from which the specific substrate had been omitted. Further controls were performed by adding 10^{-3}M physostigmine (eserine) to the incubation media.

All photographs of experimental cercariae and controls were taken using a Carl-Zeiss microscope on Ilford FP4 135 film.

Two histochemical fluorescence techniques were applied to localise biogenic amines.

5.2.4 Falck-Hillarp formaldehyde-induced fluorescence technique (FIF)

In the FIF histochemical procedure (for review on methodology, see Falck, Hillarp, Thieme & Torp, 1962; Falck & Owman, 1965; Corrodi & Jonsson, 1967; Bjorklund, Falck & Owman, 1972; Fuxe & Jonsson, 1973), four to six newly shed cercariae were placed in small drops of distilled water on a clean glass slide and then inserted horizontally in a tube. The tube was clamped in a horizontal position and connected with a freezing drying machine - Edwards high vacuum (EMFD). The cercariae were freeze dried for 20-25 minutes at -60°C under about 10^{-1} torr (i.e. 4 mbar).

At the end of the freeze-drying period, each slide was warmed up to room temperature and then kept in a desiccator over phosphorus pentoxide for two days. After that time the dried cercariae were transferred to a new desiccator containing about 5 gms paraformaldehyde in a watch glass which had been maintained at 51 to 61% RH for 10 days or more and sealed with silicone grease. The water content of the paraformaldehyde is of critical importance for the outcome of the histochemical reaction. Thus, batches of paraformaldehyde previously dried by heating at +100°C for one

hour, were stored in the desiccator at a constant relative humidity for at least 10 days before use. This was ensured by using potassium hydroxide (Solomon, 1951) in the desiccator. Previous testing on a range of humidities showed that optimum results could be obtained if the paraformaldehyde is stored with KOH solution giving a relative humidity of 51-61%.

The cercariae were then exposed to the formaldehyde vapour generated by incubating the desiccator in an oven at +80°C for one to three hours. Primary catecholamines and serotonin develop a maximum fluorescence after one to two hours, but for adrenaline it is necessary to continue the formaldehyde treatment for up to three hours. The treated cercariae were mounted in liquid paraffin, covered with a clean coverslip and examined immediately in a fluorescence microscope.

Controls were performed in which the freeze-dried cercariae were treated as described in the histochemical test but without the paraformaldehyde.

5.2.5 Glyoxylic acid (GA) fluorescence method (Bjorklund, Lindvall & Svensson, 1972; Axelsson, Bjorklund, Falck, Lindvall & Svensson, 1973; Bjorklund, Hakanson, Lindvall & Sundler, 1973; Lindvall & Bjorklund, 1974; Lindvall, Bjorklund & Svensson, 1974).

In this method, the cercariae were treated according to the procedure outlined by Lindvall & Bjorklund (1974), the procedure adopted was as follows:

Four to eight newly shed cercariae were placed in small drops of distilled water, on clean microscopic slides. Much of the excess fluid was removed with filter paper and replaced with a few drops of 2% glyoxylic acid (Sigma) in 0.1M phosphate buffer at pH 7.0 and 4°C. The cercariae were left in the solution for two to four minutes. Excess glyoxylate was blotted with filter paper and the slides dried rapidly at 60°C in warm air from a hair drier for about 15 to 20 minutes. After that, the slides were placed in a 100°C oven for five minutes, then the dried cercariae were mounted in liquid paraffin under a thin clean coverslip for immediate examination under the fluorescence microscope. Control cercariae were subjected to exactly the same procedure but without glyoxylic acid.

In both these methods, fluorescence in cercariae was observed and photographed with a Zeiss standard 14 photomicroscope which was fitted with ultra-violet light sources for both transmitted light and epi-illumination. The illumination was provided by using standard FITC excitation filter (which transmits UV light in a range of 450-490nm) and a LP 520 barrier filter.

5.3 Results

Histochemical techniques for the localisation of non-specific esterases, cholinesterases and biogenic amines all produced extensive staining of the components of the nervous system of T. patialense larvae. Although the different techniques did show some significant technique specific distributions of activity the main features of the larval nervous system were well visualised with all the techniques. The principal results from the different techniques are listed below.

5.3.1 5-bromoindoxyl acetate method (5-BrI)

Using this technique the sites of esterase activity in larvae were revealed as blue deposits of indigo granules.

In the family Transversotrematidae primordial cercariae are produced by rediae within the digestive gland of thiarid gastropods like Melanoides tuberculata and complete their development external to the rediae. Such a developmental pattern greatly facilitates the experimental isolation of individual immature cercarial larvae, permitting observation of nervous tissue in single organisms at different stages of development. Consequently, it would appear convenient to establish a series of readily recognisable stages of cercarial morphogenesis.

Four such arbitrary stages (Figure 5.1) have been designated in this way in this study, namely:

1. Type (1) cercariae (mean body width 191 μm)

These are larvae which have recently emerged from a redia. They are at first spherical or subspherical, later they assume an oval shape, with some demarcation between the body and the tail.

2. Type (2) cercariae can be conveniently divided into two sub-stages, i.e.

a) Early type (2) cercariae (mean body width 297 μm). These have

Figure 5.1 Diagram showing the sequence of cercarial developmental stages from newly emerged intraredial cercariae to mature free-living cercariae (see text for detailed structural description of the four arbitrary stages)

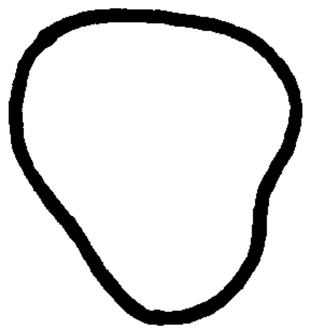
a) Type 1 cercaria

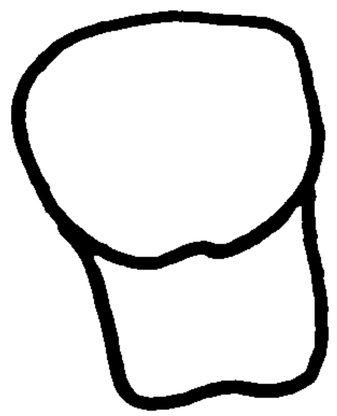
b) Type 2 cercaria

c) Type 3 cercaria

d) Type 4 cercaria

a)





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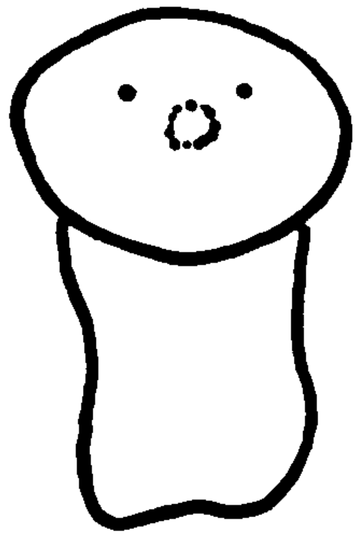
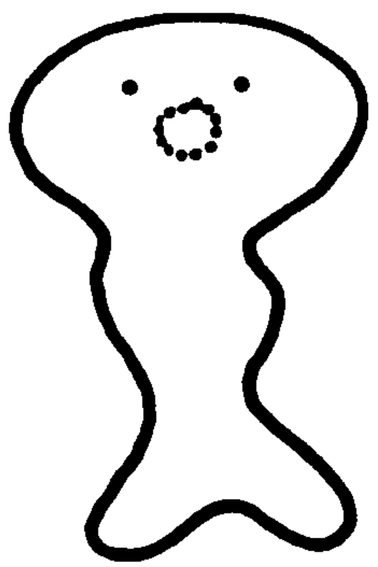
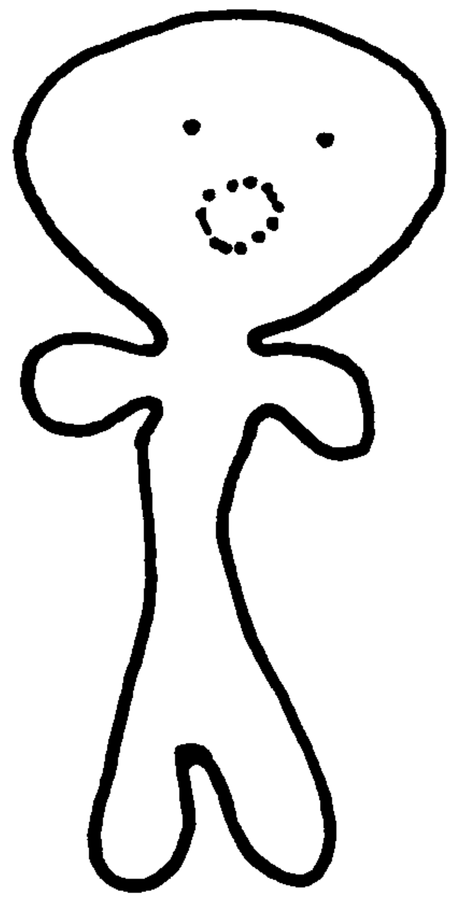
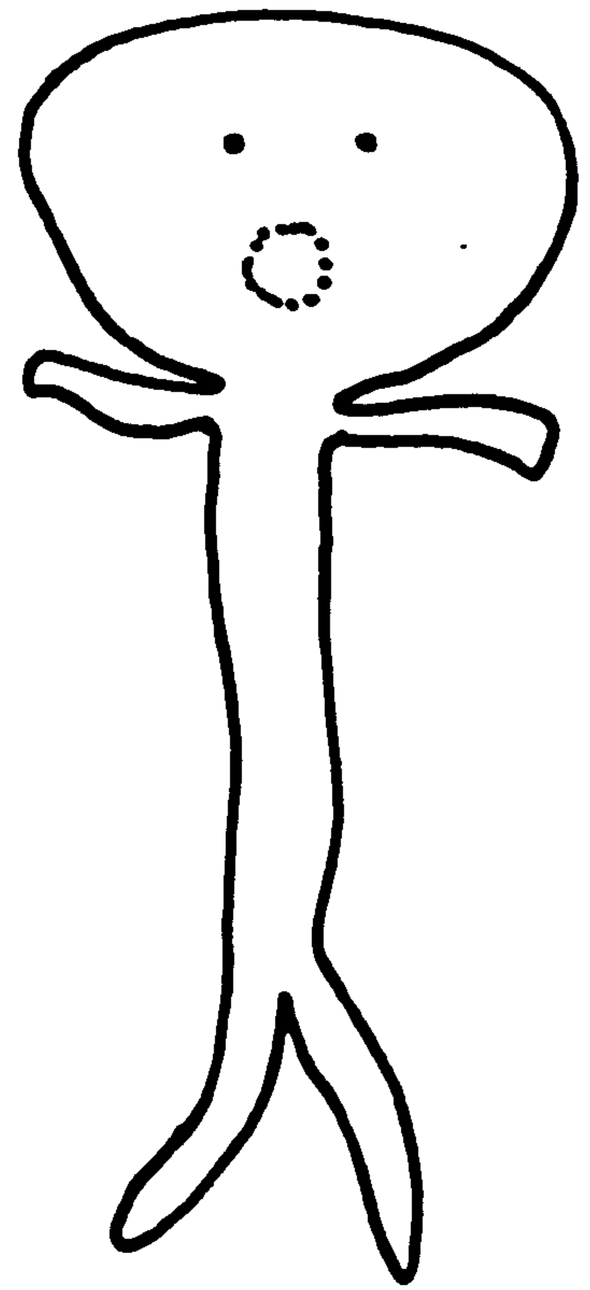




c)



d)



reached a stage of development where primordial arm processes and furcae are apparent and the ventral sucker is pedunculate. The genital pore and mouth aperture, however, remain imperforate.
b) Late type (2) cercariae (mean body width 303 μm). The development status is similar to early type (2) but with distinctly elongated arm processes.

3. Type (3) cercariae (mean body width 368 μm). These are characterised by the elongation of the arms and furcae, with a rapid growth and differentiation of the organs within the body. In particular, the genital pore and mouth aperture become perforate.

4. Type (4) cercariae (mean body width 534 μm). This is morphologically indistinguishable from free-living cercariae and shows the elongation of tail and arms and the differentiation of internal structures.

The organisation of presumptive nervous system, showing a positive reaction to this test for nonspecific esterase that could be recognised in the developing and mature cercariae was as follows: In type (1) cercariae, no evidence of nervesis apparent (Plate 5.1 A and B). In type (2)a cercariae, the nerve tissues are also not conspicuous, while in type (2)b cercariae, some preparations show a bilateral anterior concentration of positively staining, presumably neural, tissue (Plate 5.1C), while others showed a longitudinal tract in the middle of the tail and a small granular deposit scattered over the whole cercariae (Plate 5.1D).

In type (3) cercariae, two bilaterally symmetrical anterior concentrations of neural tissue are apparent as are two longitudinal tracts that pass into the tail. Several nerve branches are given off laterally and one medially from the main longitudinal tracts (plate 5.2A). In type (4) cercariae (Plate 5.2B), two main longitudinal tracts are situated lateral to the ocelli, each of them thickened slightly anteriorly. The tracts are connected by a commissure anterior to the oral sucker and another posteriorly near the body tail junction. Well developed nerve branches extend anteriorly, laterally and posteriorly taking origin from the main longitudinal tract. Two other branches arise near the ocelli and directed posteriorly forming a nerve plexus over the ventral sucker, then pass downward to join the main longitudinal tract near their posterior junction .

A prominent dense area of reaction product is found at the body tail junction from which two longitudinal nerve tracts arise and pass down the tail, uniting posteriorly at the base of the furcae forming a bikbed neural mass, From each lobe of this mass, two nerve cords pass into the corresponding furca.

For the bromoindoxyl acetate staining, three types of controls were carried out on the developing larval stages and free-living cercariae. Firstly, larvae were heated to 90°C for five minutes prior to incubation to denature relevant enzyme. Secondly, larvae were incubated in media in which the substrate had been omitted, and thirdly they were incubated in medium including 10^{-3} M. physostigmine (eserine) as an inhibitor of acetylcholinesterase.

In each of these three control contexts, no reaction product was produced in the nervous tissue areas of larvae of any developmental stage. This finding in respect of the eserine control material suggests strongly that most if not all the positive reactions obtained with this histochemical technique within T. patialense cercariae must be due to acetylcholinesterase activity.

5.3.2 α-naphthylacetate method (αNA)

This technique although less specific in its responsiveness than the bromoindoxyl acetate technique, has proved very useful in demonstrating the general organisation of the nervous system in T. patialense. The positive reaction appears as a dark brown deposit of dye which is somewhat more diffuse than in the other methods.

In type (1) cercariae, no nerve tissue could be detected in the newly emerged cercariae from rediae, while in late type (1) cercariae, a bilaterally symmetrical diffuse area of moderate reaction was detected anteriorly (Plate 5.3A).

In early type (2) a , intramolluscan cercariae, the developing nervous tissue appeared as two bilaterally symmetrical anterior concentrations of nervous tissue with two main longitudinal tracts continuing into the tail, with some evidence of a fine transverse anterior link between the two main nervous concentrations. No side branches are apparent. (Plate 5.3B).

In late type (2) b, intramolluscan cercariae, there was some evidence of anteriorly directed branches originating from the nerve mass and the longitudinal tract (Plate 5.3C).

In type (3) intramolluscan cercariae, anterior, lateral and posterior, outwardly directed branches of the main tracts were apparent. In addition, development of the main branches which originate from the inner side of the nerve masses was in progress and the beginning of the formation of the posterior link between the two main longitudinal tracts was seen. At the same time, a dense nerve mass near the body tail junction was prominent. (Plate 5.3D).

In type(4) intramolluscan and free-living cercariae (Plate 5.4A), the nervous system consisted of two longitudinal tracts situated laterally on each side of the ocelli, each with a slight thickening anteriorly and connected anteriorly by a transverse commissure which is curved over the mouth opening. Posteriorly, the two longitudinal tracts were linked together by a fine commissure. Eight pairs of outer branches originated from the two longitudinal tracts directed anteriorly, laterally and posteriorly. Most of them broke up into fine branches distal to the main tract. One pair of branches arose from the inner side of the thick portion of the longitudinal tract and extended backwards joining the posterior link between the two longitudinal tracts. In some preparations posterior to the ventral sucker, the two inner branches united into one branch that joined the posterior transverse commissure. In the region of the ventral sucker, the inner branches divided into fine arborizations posterior to the oral sucker forming a nerve plexus which along with the anterior transverse commissure anterior to the oral sucker constitute a circumoesophageal ring. Two pairs of transverse commissure joining the inner branches with the main longitudinal tract were observed. At the body-tail junction a dense area of black deposit was observed, giving rise to two longitudinal tracts running down through the tail and ending in a bilobed nervous mass from which two nerve cords passed into each furca.

In controls, complete inhibition of the reactions in the nervous tissue were achieved in the larval stages and mature free-living cercariae by the three types of controls utilised in this experiment. This would strongly suggest that the enzyme hydrolyzing the acetate substrate is a cholinesterase. (Plate 5.4B).

5.3.3 Acetylthiocholine iodide method (ATCI)

The extent to which the mature free-living cercariae were stained by the application of this technique was quite variable, most of the cercariae showed small localised reddish-brown deposits on the surface of the body, while the deposit in the tail was quite consistent. However, in some preparations, an intense reaction was detected in the nervous tissue which was identical to the picture of the nervous system obtained from ∝naphthyl acetate histochemical method (Plate 5.5A).

In controls, the inclusion of 10^{-3}M. eserine in the incubation medium completely abolished the reaction. No reactions were obtained after incubation in media lacking the specific substrate, and all the activity was eliminated by exposure to 90°C for five minutes before incubation. (Plate 5.5B).

5.3.4 Falck-Hillarp Formaldehyde-induced fluorescence technique (FIF)

The application of FIF method revealed a distribution of fluorescent structures in the nervous tissue of cercariae (Plate 5.6A and B) which was not observed in controls which had not been treated with formaldehyde (Plate 5.8B).

Green fluorescence was generated by exposure to formaldehyde vapour at 80°C for one hour. Exposure for longer periods (three hours) did not result in any noticeable intensification of fluorescence.

Fluorescence was distributed in the nervous tissues of cercariae in a manner broadly corresponding to the pattern of esterase activity in the nervous system (Plate 5.5A). In addition to these areas of fluorescence, however, several bright and discrete, fluorescent spots were obvious in most of the specimens examined. These bright spots were located within or adjacent to the main nerve tracts and its branches and beneath the ocelli within the inner nerve branches. (See Figure 5.2)

5.3.5 Glyoxylic acid fluorescence method (GA)

Application of GA method to cercariae have shown that the distribution of green fluorescence (Plate 5.7A) appeared more or less similar to that observed with the FIF method. However, the fluorescence was visualised only in the main branches of the nervous system in most of the preparations and the inner branches were less conspicuous. The brightly fluorescent, discrete spots were not easily detected in most specimens examined (Figure 5.3).

Figure 5.2 Diagrams showing the distribution of fluorescent, presumably catecholamine-containing, cells in the nervous structures of the head of cercariae (FIF method)

Each diagram illustrates the pattern in an individual cercaria. Fluorescent cells are shown in black and the open system represents the main tracts of the nervous system of the head.

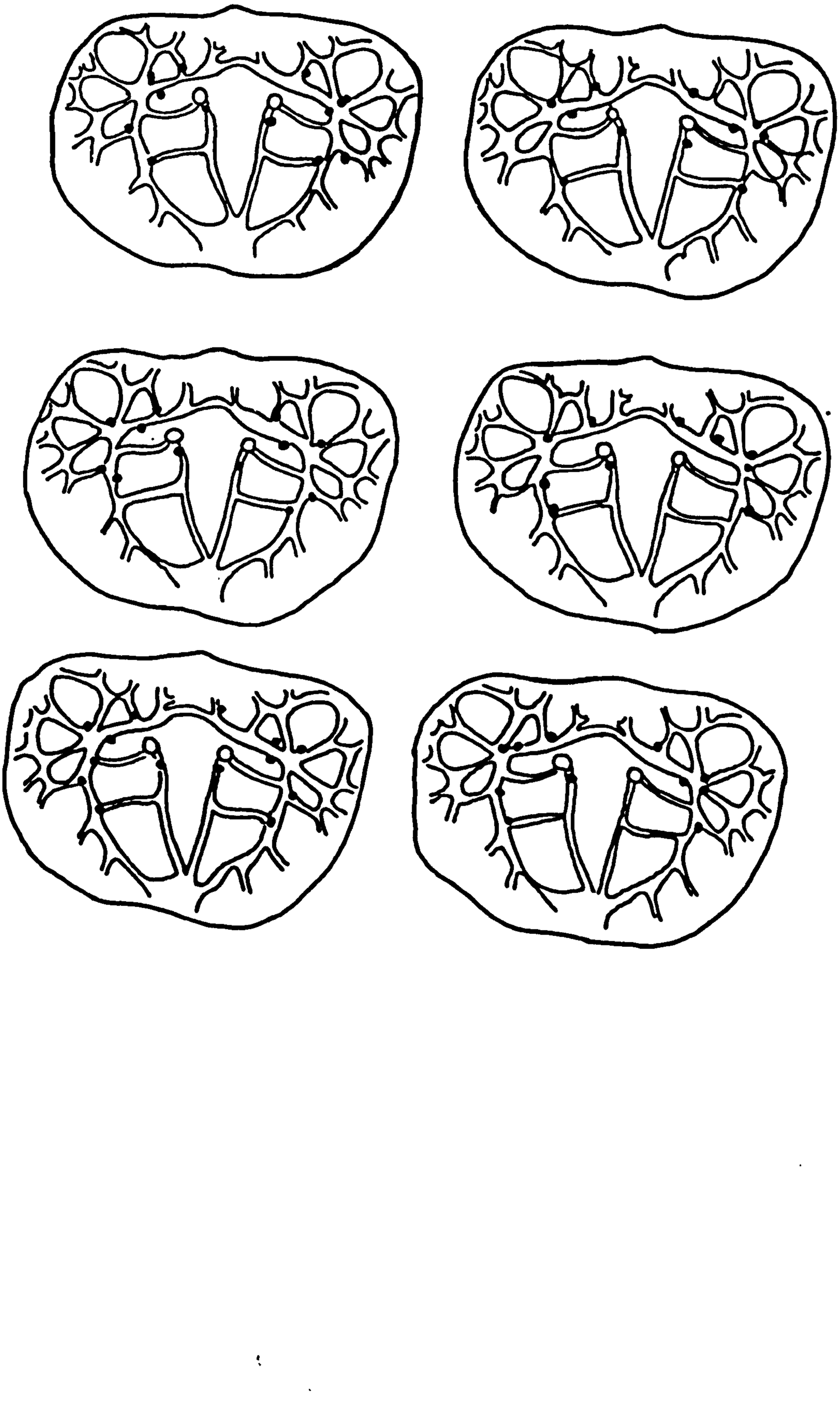


Figure 5.3 Diagrams showing the distribution of fluorescent, presumably catecholamine-containg, cells in the nervous structures of the head of cercariae (GA method)

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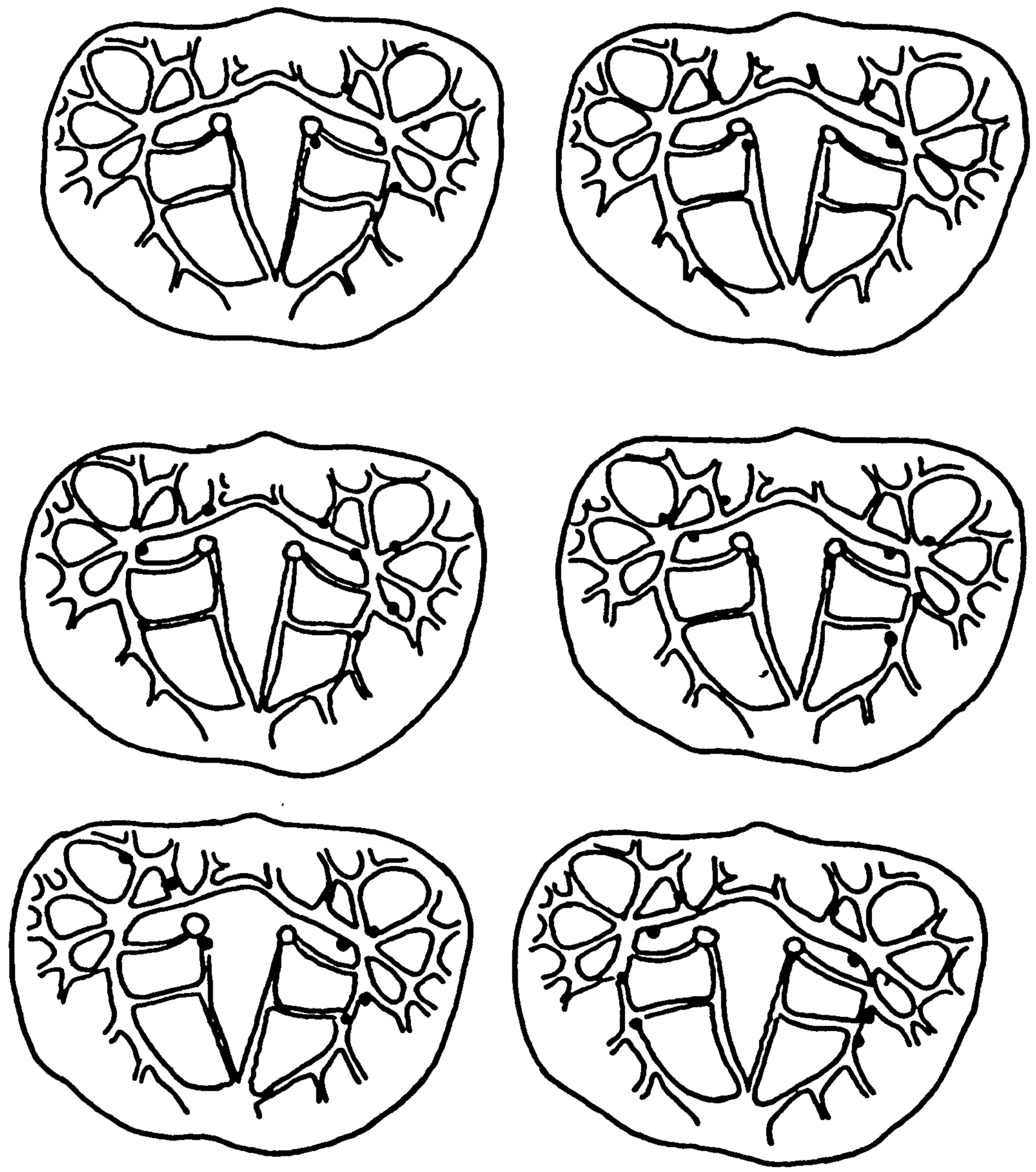


Both these effects may be due to diffusion of the bioamines from the nervous tissue, since the GA treated cercariae were only air-dried and the degree of humidity is an important factor in generating the fluorescence. In addition to the fluorescence in the body, two longitudinal varicose tracts were observed running along the tail (Plate 5.7B) and a high level of fluorescence was sometimes observed in parts of the male genital organs (Plate 5.8A). No significant nervous tissue-related fluorescence was observed in the untreated cercariae (Plate 5.8C).

5.3.6 Description of the cercarial nervous system at the light microscope level

A consistent general pattern of nervous organization emerges from a comparison of the finding obtained with the different histochemical techniques which have been utilised in this study.

5.3.6.1 The structure of the nervous system during development from intramolluscan cercariae to mature cercariae

The different histochemical findings and the results obtained from the examination of developing cercariae have enabled a basic morphological interpretation of the development and final structure of the cercarial nervous system of T. patialense to be built up.

5.3.6.2 Development

Rudiments of the nervous system are present early in development. In late type (1) intramolluscan cercariae, a bilaterally symmetrical diffuse area at the anterior end is present on each side. These zones represent primordial cerebral ganglia. As development proceeds, these two nerve masses become more prominent and concentrated with two main longitudinal tracts extending from them into the tail with a fine transverse anterior link between the two nerve masses being apparent in early type (2) intramolluscan cercariae. As the arms and furcae become more obvious, outer branches differentiate from the main longitudinal tracts. With further development of the cercariae a complete sequence of outer branches forms and two branches originate from the inner side of the nerve masses running down the body. A posterior link between the two main longitudinal tracts then forms and a dense nerve mass near the body-tail junction appears.

5.3.6.3 The mature cercarial nervous system

The nervous system of T. patialense cercariae (Figure 5.4) is composed of a pair of longitudinal nerve tracts that pass laterally joining together near the body-tail junction. They probably represent the posterior dorsal nerve cords. The anterior thickennings of these nerve tracts which pass anteriomedially in front of the ocelli probably represent the cerebral ganglia which are united by a narrow transverse commissure curving anterior to the mouth.

A pair of branches arise medially from the thick anterior portion of the longitudinal tracts and extend backwards forming an internal nerve plexus situated posterior to the oral sucker which represents a portion of a circumoesophageal nerve ring. Thereafter, these internal nerve branches continue posteriorly past the ventral sucker forming the posterior ventral nerve cords. These fuse together and join the posterior dorsal nerve cords at the posterior end of the body. In some preparations the posterior ventral nerve cords appear to unite below the ventral sucker into one posteriorly directed branch which joins the posterior junction.

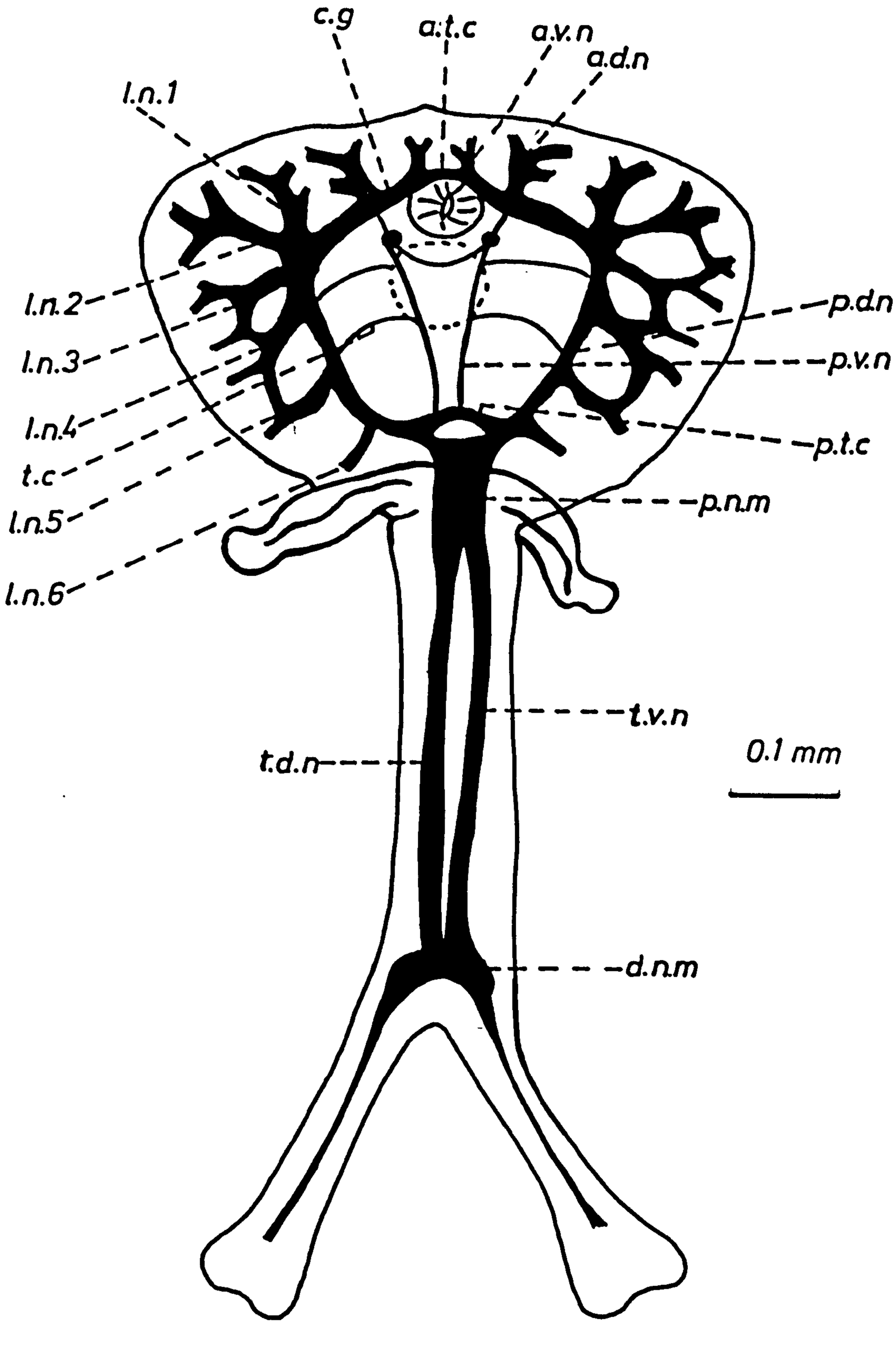
Eight pairs of outwardly directed nerve branches originate from the cerebral ganglia and the presumptive dorsal longitudinal tracts and pass anteriorly, laterally and posteriorly from them.

Two anterior nerve branches arise symmetrically from the most anterior regions of each cerebral ganglia. The inner pair shortly after their origin give off a side branch to the central zone of the anterior region (probably close to the genital atrium). Thereafter, they pass anteriorly for short distance and divide into fine branches. The outer pair are much more conspicuous than the inner pair. Each of these branches also gives off a side branch laterally, then splits into two.

Six lateral pairs originate from the dorsal longitudinal tracts themselves. Three pairs arise close together at the junction of the tracts with the thickened cerebral ganglia, anteriolaterally, laterally and posterolaterally respectively. The remaining other pairs arise along the middle and posterior part of the nerve cords, on each side. The total complement of outer branches is as follows : Nerve (1) is directed somewhat superiorly, giving off a side branch and divided into two short

Figure 5.4 Diagrammatic representation of the nervous system of a mature free-living T. patialense cercaria

adn: anterior dorsal nerve; atc: anterior transverse commissure; avn: anterior ventral nerve; cg: cerebral granglion; dnm: distal nerve mass; ln 1,2,3,4,5,6: lateral nerve number 1,2,3,4,5,6; pdn: posterior dorsal nerve; pnm: proximal nerve mass; ptc: posterior transverse commissure; pvn: posterior ventral nerve; tc: transverse commissure; tdn: tail dorsal nerve; tvn: tail ventral nerve


c.g
a.t.c
a.v.n
a.d.n
l.n.1
l.n.2
l.n.3
l.n.4
t.c
l.n.5
l.n.6
p.d.n
p.v.n
p.t.c
p.n.m
t.v.n
t.d.n
d.n.m
0.1 mm

branches.

Nerve (2) is divided into anterior and posterior branches with a small branch from the posterior one.

Nerve (3) is also divided into anterior and posterior branches, the anterior branch linked with the posterior branch of nerve (2).

Nerve (4) is divided into anterior and posterior branches, the anterior branch linked with the posterior branch of nerve (3). The posterior branch is directed backward with a short side branch.

Nerve (5) joins with the posterior branch of nerve (4).

Nerve (6) is a small unbranched nerve.

The presumed postero-ventral and postero-dorsal nerve cords are connected by two transverse commissures, one anterior to the ventral sucker and the other near the posterior third of the ventral sucker.

At locations near the cerebral ganglia, the main nerve cords and the origins of the main nerve branches, large brightly fluorescent spots (about 6μm in diameter) are apparent when histochemical techniques for catecholamines are employed. These spots are interpreted here as bearing individual catecholamine-containing nerve cell bodies. They occur in relatively constant positions with relation to the general framework of the central nervous system.

Close to body-tail junction there is a nervous mass from which dorsal and ventral median nerve cords extend the length of the tail stem beneath the subtegumental muscle layer. These are joined at the proximal end of the tail by a dorso-ventrally oriented commissure, and distally they end in a bilobed nerve mass from each side of which two nerve cords run into the corresponding furca.

5.4 Discussion

The results presented in this chapter provide a map of the cercarial nervous system of T. patialense related to two different types of histochemical localization. One pattern of localization is the consequence of esterases located within the nervous system. Although both nonspecific and relatively specific substrates for esterases were employed, the fact that 10^{-3}M physostigmine (eserine) abolished almost all non-specific esterase activity suggests that all the techniques

employed were in fact demonstrating cholinesterase activity. The other map of the nervous system is one concerned only with the distribution of biogenic amines as revealed by fluorescence methods.

In many respects it is remarkable that the two nervous system descriptions, i.e. those based on cholinesterase and biogenic amine distributions, should be so similar. Despite differences in very detailed appearance, the overall picture of the nervous system provided by these two quite different sets of techniques are very alike. The only marked difference concerns the visualization of brightly fluorescent spots, interpreted here as catecholamine-containing cell bodies, by the fluorescence techniques. Their presence would have been unsuspected with the use of esterase techniques alone.

Despite the general compatabilities of the interpretations of nervous system structure from the different methods, some of the differences that did emerge deserve further analysis.

In general, the reactions obtained with the 5.BrI and∝NA methods for nonspecific esterases with whole mounts of developing and mature cercariae were characterised by intense activity in the nervous tissues. With 5.BrI the blue precipitate was finely localized. While with∝NA considerable diffusion of the dark brown dye occurred, demonstrating the main nerve tracts clearly but obscuring finer details. The substrates ∝NA and 5.BrI are hydrolised by nonspecific carboxylic-esterase complex, the constituents of which may be characterised by their response to various inhibitors (Pearse, 1960). However, as mentioned above, the reactions were almost totally abolished by 10^{-3}M physostigmine (eserine) which strongly suggested that the enzymes hydrolising the substrates were not arom-esterase, ali-esterases, C-esterase, or lipase (see Pearse, 1960), but consisted entirely of cholinesterases.

The acetylthiocholine iodide (ATCI) method, a technique of considerable specificity for cholinesterase, does selectively demonstrate elements of the nervous system in about 10% of the preparations made. Positive reactions appeared as reddish brown microcrystals. In the heads of most cercariae the reaction appeared as small dark spots of different sizes within the tissues but also on the surface of the body. Tails showed a more

consistent intensa reaction within nervous system components.

It seems, perhaps, that differential penetration of substrates is taking place in the two parts of the body and that this permeability difference may be due to a difference in the properties of the surface of the cercariae.

Bruckner & Voge (1974) and Nuttman (1975) were faced with the same problems when analysing ATCI localization in Schistosoma mansoni cercariae. They found that when using this method, stain appeared as a small localized deposit on the surface of the organism with some heavier staining at the anterior and posterior ends of the body. The former authors described the deposits of the stain on the surface of the body as appearing under high magnification as a minute volcanic crater with a relatively dark centre surrounded by a black ring. These deposits of stain would appear to represent positive reactions associated with ciliated tegumental receptor endings. Both workers concluded that ATCI did not penetrate through the cercarial body surface and that the surface permeability should be altered to permit penetration and staining of internal structures. Nuttman (1975) suggested that part of the cercarial surface (probably the neutral polysaccharide glycocalyx coat) is relatively impermeable to the substrate used for cholinesterase localization. It does seem, in this context, that in T. patialense cercariae the surface of the tail is almost always permeable to ATCI as in nearly all cercariae the nervous structures of the tail could be visualized with this technique. The far less consistent results obtained for heads perhaps suggested that the permeability of head and tail teguments are different. As the tail only operates in fresh water conditions, but the head later becomes adapted to higher ionic concentrations under the fish scales (Mills, 1979a) such a difference is quite plausible.

The FIF method clearly demonstrated all the major components of the cercarial nervous system. The GA method was unable to show the fine detailed nervous structures possible with FIF in all the specimens examined, although the intensity of the fluorescence induced was greater than that associated with the FIF method. This sort of difference in the intensity as revealed

by the two methods was also reported by Lindvall & Bjorklund (1974). Variability in the localization of fluorescence in different specimens may be due to the degree of stretching of the cercariae and also to the degree of dryness of the cercariae being variable. GA treated cercariae are only air-dried (water than freeze dried) and incomplete dryness causes the diffusion of fluorophores (Lindvall & Bjorklund, 1974).

A bilaterally symmetrical nervous system in mature free-living T. patialense cercariae has been demonstrated in the present study. This is a result which conforms in a general way to the basic digenean pattern of nervous tissue arrangement which is characterised by two cerebral ganglia connected by transverse commissures dorsal and ventral to the gut and three or four anteriorly and three or four posteriorly directed pairs of nerve tracts originating from them.

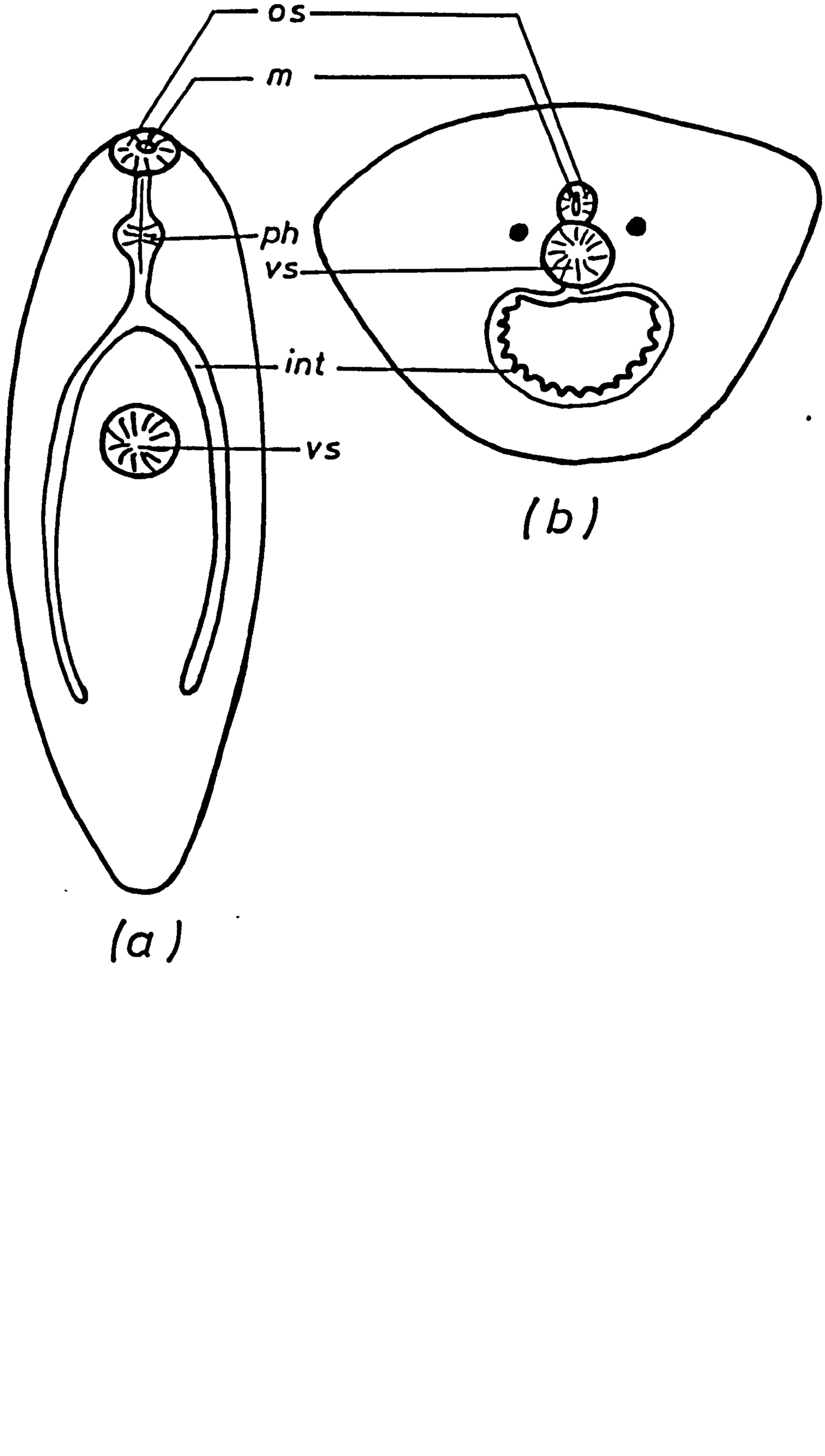
In T. patialense cercariae, however, the anatomy of the nervous system, especially the arrangement of the nerve branches, is modified in comparison with other digenean cercariae (see, for instance, Plagiorchis (Multiglandularis) megalorchis, Rees, 1952; Parorchis acanthus, Rees, 1967; Schistosoma mansoni, Fripp, 1967; Bruckner & Voge, 1974; Plagiorchis elegans, 1979; Cloacitrema michiganensis, Leflore, Bass & Smith, 1980; Haplometra cylindracea, Grabda-Kazubska & Moczon,1981; Diplostomum pseudospathaceum, Niewiadomska & Moczon, 1982). This modification is probably attributable to the shape of cercarial head which itself is the same as that of the adult fluke. It is characterised by a laterally expanded body to provide a scale-like shape since the adult is an ectoparasite, inhabiting the recesses beneath the scales of tropical fresh-water fish. During the assumption of this shape the body has become wider than it is long in contrast to the reverse conditions in about all other digenean groups.

In general, the digeneans have an elongated oval form (Figure 5.5) although there are few which are rounded or disc like and the blood-inhabiting flukes tend to be slender and thread-like. The body usually tapers at the anterior end, which is not marked off as a head and the mouth is usually situated at or near the anterior end encircled by an oral sucker.

In T. patialense, however, the mouth has moved posteriorly

Figure 5.5 Diagrams illustrating the differences in body form between a typical digenean adult (a) and an adult fluke of T. patialense (b)

int: intestine; m: mouth; os: oral sucker; ph: pharynx; vs: ventral sucker


os
m
ph
vs
int
vs
(b)
(a)

along the ventral surface to become localized between the anterior edge of the body and the mid-ventral ventral sucker. This change in location when compared with most other digeneans (see Figure 5.5) is presumed to be associated with the surface browsing- feeding activities of the adult fluke (Whitfield, 1979).

The two principal peculiarities, therefore, of the body plan of the cercarial head of T. patialense are firstly a laterally expanded form and secondly the ventral position of the mouth opening. These two changes appear to be the factors which have resulted in the transversotrematid nervous system plan departing from the normal digenean one with its compact cerebral ganglia behind the mouth.

In T. patialense we find no demarcated discrete cerebral ganglia. Instead, the regions interpreted here as cerebral ganglia are elongate thickened tracts of nervous tissue which merge into somewhat thinner posterior nerve tracts behind. This apparent expansion of the cerebral ganglia region appears to be correlated with the lateral expansion of the body itself. It may be assumed to bring nerve branches close to the lateral zones of, for instance, body muscles.

The cerebral ganglia region of T. patialense cercariae, however, is not only aberrant in its shape but also in its location, linked by a presumed dorsal commissure the ganglia are located in front of and lateral to the mouth cavity. This configuration is possibly unique amongst the digeneans and is undoubtedly the result of the relatively posterior position of the mouth when compared with other digeneans.

From the cerebral ganglia a well-developed pair of longitudinal nerve tracts pass laterally and join together near the body-tail junction. It is concluded here that these represent the posterior dorsal nerve cords. In most other digenean cercariae and adults it is the posterior ventral nerve cords which are the most distinct. This difference if it is a real one would again be the result of the posterior mouth opening. Without, for instance, serially sectioning cercarial heads at an ultrastructural level, it is difficult, however, to be absolutely sure that the posterior cords are dorsal ones. The changed conformation of the cerebral ganglia make such an analysis very difficult.

The presumed posterior dorsal and ventral longitudinal nerve cords of cercariae terminate at the posterior end of the head and join together there. In this respect, the nervous system of T. patialense cercariae resembles that of the cercariae of Schistosoma mansoni (Bruckner & Voge, 1974); Plagiorchis elegans (Leflore, 1979); Cloacitrema michiganensis (Leflore et al.,1980); Haplometra cylindracea (Grabda-Kazubska & Moczon, 1981); Diplostomum pseudospathaceum (Niewiadomska & Moczon, 1982) using histochemical methods.

As far as the arrangement and structure of the transverse commissures between longitudinal nerve cords are concerned, only two transverse commissures were found joining the posterior longitudinal nerve tracts in T. patialense. While in other digenean cercariae several anterior and posterior transverse commissures have been observed (see Grabda-Kuzubska & Moczon, 1981; Niewiadomska & Moczon, 1982).

A particularly interesting feature of the cercarial nervous system of T. patialense is the intense localization cholinesterase activity in its tail. Such localization demonstrated the nervous structure in the tail as consisting of dorsal and ventral median nerve cords connected indirectly to those of the body through an area of intense NSE and AChE localization near the head-tail junction which probably represents a proximal caudal nervous mass.

The two ventral and dorsal nerve cords fuse posteriorly in the tail to form a bilobed nervous mass from which two nerve cords run on each side into the corresponding furca.

As will be shown later (see Chapter 6), the tail of a T. patialense cercaria has intrinsic locomotory abilities. Detached tails are able to continue in coordinated rapid swimming activity despite the lack of nervous connectivity with the central nervous system in the head. Such autonomous patterned behaviour strongly suggests that the tail itself contains controlling nervous elements with coordinatory and/or pacemaker abilities. The proximal and distal nerve masses of the tail of T. patialense cercariae are the best candidates for these roles.

Localization of cholinesterase activity at the body-tail junction has also been reported in other cercariae (see, for example, Fripp, 1967; Leflore, 1979; Leflore et al.,1980).

However, Leflore (1979) and Leflore et al,(1980) enumerates three pairs of longitudinal nerve cords in the tail of Plagiorchis elegans and Cloacitrema michiganensis respectively corresponding to those of the body. In contrast, only one nerve trunk was recovered in the tail of Haplometra cylindracea (Grabda-Kuzubska & Moczon, 1981) and two nerve trunks in Diplostomum pseudospathaceum (Niewiadomska & Moczon, 1982).

As mentioned above, the results of control histochemical experiments in which 10^{-3}M physostigmine (eserine) was used as an inhibitor suggest most strongly that the esterase activity demonstrated in T. patialense cercariae are of the cholinesterase type.

Eserine has been found to inhibit esterase activity in the nervous system of all the digenean cercariae in which its inhibitory activity has been investigated (Schistosoma mansoni, Fripp, 1967; Bruckner & Voge, 1974; Himasthla quissetensis; Zoogonus lasius, Jennings & Leflore, 1972; Plagiorchis elegans, Leflore, 1979; Cloacitrema michiganensis, Leflore et al., 1980; Haplometra cylindracea, Grabda-Kazubska & Moczon, 1981; Diplostomum pseudospathaceum, Niewiadomska & Moczon, 1982).

These findings, taken together, mean that it is difficult not to conclude that many, if not all, cercariae possess cholinergic chemical transmission between nervous elements or between nerves and muscles.

Acetylcholinesterase associated with the nervous system has been demonstrated by histochemical methods by other workers in other digenean species. In Schistosoma mansoni miracidium (Papler, 1958; Fripp, 1967; Bruckner & Voge, 1974) cercariae (Lewert & Hopkins, 1965; Fripp, 1967; Bruckner & Voge, 1974; Nuttman, 1975) adult (Fripp, 1967; Bueding, Schiller & Bourgeois, 1967). In Schistosoma rodhaini (Fripp, 1967), in Schistosoma haematobium and Schistosoma japonicum (Bueding et al, 1967), in Fasciola hepatica and Dicrocoelium dendriticum (Ramisz & Szankowska, 1970).

It has also been demonstrated in cestode species (Lee, Rothman & Senturia, 1963; Lee & Tatchell, 1964; Schardein & Waitz, 1965; Arme, 1966; Bogitsch, 1965; Hart, 1967; Ohman-James, 1968; Shield, 1969; Wilson & Schiller, 1969).

The presence of AChE has been chemically confirmed by Bueding, 1952 in Schistosoma mansoni; Chance & Mansour, 1953, in Fasciola hepatica; Schwabe, Koussa & Acra, 1961, in Echinococcus granulosus cyst and Graft & Read, 1967, in Hymenolepis diminuta by homogenizing and extraction of the specimens and demonstrating such activity in the homogenates.

The application of the fluorescence histochemical methods to T. patialense cercariae has permitted the localization of substances presumed to be catecholamines in the nervous system of this parasite. According to Falck, et.al. 1962, condensation of biogenic amines with formaldehyde forms a highly specific fluorescence product.

A yellow fluorescence appears after exposure to formaldehyde vapour at 80°C for one hour for the presence of indolealkylamine (i.e. 5HT). While the green fluorescence under the same conditions indicates the presence of primary catecholamines such as dopamine and noradrenaline. The appearance of yellow or green fluorescence after treatment by more severe reaction conditions (three hours exposure at 80°C) indicates the presence of secondary catecholamines such as adrenaline (Corrodi & Jonsson, 1967).

The green fluorescence observed in T. patialense cercariae probably indicates the presence of catecholamines in the nervous system. This is a result produced by both the FIF method and GA histochemical technique. It was impossible, however, to know whether the green fluorescent amine reaction product in the nervous system of cercariae represented any particular catecholamine or combination of them. Precise identification of the amines responsible for the fluorescence will require use in the future of various inhibitors of the reaction of the amines with formaldehyde or GA and cytospectrofluorometric analysis.

It is worth mentioning here again that fluorescence appeared in the seminal vesicle and testes of cercariae subjected to the GA method but not with the FIF technique. This fluorescence may be partly due to autofluorescence. It seems likely, though, that it must be also partly the results of biogenic amines in these organs, either in the organs themselves or in the nerves which innervate them.

The results of the FIF and GA techniques lead to the conclusion that the nervous system of T. patialense must utilize catecholamines

as well as cholinergic chemical transmitters. Recently Bennett & Gianutson (1977) demonstrated catecholamines containing neuronal structures distributed throughout the immature Fasciola hepatica. They reported that catecholamine containing cells (possibly neurones) and fibres were more prominent in the anterior region of the body and that dopamine was the only catecholamine identifiable in an extract of immature flukes.

A comparative discussion of the evidence for such adrenergic neural activity in other parasitic platyhelminths and invertebrates will be undertaken in Chapters 6 and 8. It is important to note, however, that a similar simultaneous localization of biogenic amines and acetylcholinesterase has been reported in Schistosoma mansoni (Bennett & Bueding, 1971; Machado, Machado & Pellegrino, 1971; Bueding et.al.,1967) in cestode Dipylidium caninum (Shield, 1969, 1971), Hymenolepis diminuta and Hymenolepis nana (Wilson & Schiller, 1969; Lee et al.,1978); Diphyllobothrium dendriticum (Gustafsson & Wikgen, 1981; Ohman-James, 1968).

An unusual finding of this study has been the demonstration of catecholamine-positive spots associated with the periphery of the cerebral ganglia and main nerve cords and branches of the cercarial head. It seems that such a localization of fluorescent reaction product has not been observed previously in parasitic platyhelminths. The spots are demonstratable with either the FIF or GA techniques, have fixed bilaterally symmetrical positions and are each about 6μm in diameter. These facts all support the conclusion that the spots represent the cell bodies of nerve cells that contain catecholamine inclusions. All nerve cell bodies observed ultrastructurally in this study (see Chapter 8) are positioned close to the immediate periphery of the nerve fibre meshwork of the neuropile of ganglia or nerve tracts. The bright spots are exactly in this type of position. The more diffuse, and less intense fluorescence of the neuropile of the cerebral ganglia and the other nervous components of the cercariae are presumed to be the result of axons and dendrites arising from these cells which contain catecholamines. It seems quite possible (see Figure 5.2) that all of the catecholamine related fluorescence in the cercarial head is the result of no more than about 14 nerve cells of this type.

CHAPTER 6

The effect of pharmacological agents on the activity of T. patialense cercariae

6.1 Introduction

In the great majority of digenean life cycles, only the miracidial and cercarial developmental stages lead active free-living existences. That of cercariae, although short in absolute terms, has vital significance, transmitting as it does the infection from the first intermediate host to the next host in the life cycle, which may be the final host or a second intermediate host. In the case of transversotrematid life histories as is the case in most other digenean examples, the cercariae actively swim to reach their next host.

This free-living swimming existence of T. patialense cercariae has been described in general terms by Anderson & Whitfield (1975) and in greater detail by Whitfield, Anderson & Moloney (1975) and Whitfield, Anderson & Bundy (1977). The cercarial activity before attachment to a fish host can be divided into three phases: active swimming, passive dropping and resting. During bursts of active swimming, the larvae swim tail first, with the head region folded back ventrally at the head-tail junction and wrapped around the base of the tail. Bursts of swimming can follow paths which are approximately straight, helical or highly irregular in direction. Normally, however, these paths have an upward vertical component. The free-living phase of activity ends when either the cercariae die or attach to a definitive host.

This chapter describes the results of attempts to gain insights into the control of cercarial behaviour in T. patialense by investigating the action of externally applied pharmacological agents to groups of unconstrained cercariae. The experimental systems utilised were only capable of monitoring inhibitions of cercarial activity by drugs. Direct demonstrations of a drug-related increases in activity are extremely difficult to achieve using the cercariae of T. patialense. These larvae, during the first few hours of their free-swimming existence are potentially continuously active. Thereafter the situation is made difficult to analyse because the cercariae have reduced nutrient reserves.

All the drugs used on T. patialense cercariae were known to have effects on nerve and muscle systems in other animals. Despite the problems inherent in interpreting the results of experiments which expose whole organisms to drugs, it was felt that the almost total absence of pharmacological information about digeneans justified the attempt. The range of drugs utilized consisted of substances known to act as transmitters in other systems as well as transmitter receptor inhibitors and inhibitors of acetylcholinesterase. The general significance of the pharmacological agents which were used in this study are outlined below:

6.1.1 Adrenaline

Adrenaline is a substance involved in autonomic neurohumoral transmission (Goodman & Gilman, 1980). It is found together with small amounts of noradrenaline in the chromaffin tissue of the adrenal medulla and is present in small quantities in adrenergic nerve fibres (Lewis, 1965). The main biological function of adrenaline as a hormone is to place the organism rapidly in a position in which it can best cope with an adverse situation. In animals adrenaline may have stimulant or depressant actions on the central nervous system (Leake & Walker, 1980).

6.1.2 Noradrenaline

Noradrenaline is the adrenergic nerve transmitter in vertebrates. It is also found in the suprarenal medulla of the adrenal gland but in small quantities. It is synthesized in peripheral and central adrenergic neurons and in the chromaffin cells of the adrenal medulla. It can act in three ways, as the adrenergic transmitter, as a precursor in the biosynthesis of adrenaline and as a hormone (Lewis, 1965). Noradrenaline appears to play, at most, a very minor transmitter role in invertebrate animals (Leake & Walker, 1980). Twarog & Roeder (1957) found that high concentrations of noradrenaline block transmission at the sixth abdominal ganglion of the cockroach Periplanta americana.

6.1.3 Dopamine

Dopamine is the immediate metabolic precursor of both noradrenaline and adrenaline. Dopamine is a central neurotransmitter in vertebrate animals and possesses important intrinsic pharmacological properties (Goodman & Gilman, 1980).

It also plays a major role as a transmitter at invertebrate central synapses. In insects dopamine has an important function in the hardening and tanning of the insect cuticle (Kerkut, 1973). Dopamine has a strong inhibitary effect on the stretch receptor neurons of the crayfish Pacifastacus (McGeer, McGeer & Mclennan, 1961) and on the heart beat of the limpet Patella vulgata (Leake, Evans & Walker, 1971). Ascher (1972) found that dopamine can induce inhibition, excitation or a biphasic (excitatory-inhibitory) response in Aplysia neurons. Dopamine, adrenaline, noradrenaline and related compounds are collectively called catecholamines (Lewis, 1965).

6.1.4 5-Hydroxytryptamine (Serotonin; 5HT)

5-Hydroxytryptamine is widely distributed in the animal and plant kingdoms and possesses a variety of pharmacological actions (Goodman & Gilman, 1980). The major function of 5HT is to serve as a neurotransmitter in vertebrates and invertebrates (Leake & Walker, 1980). It is also found in protozoa (Janakidevi, Dewey & Kidder, 1966) and in blood platelets (Pletsher & Daprada, 1975). 5HT stimulates or inhibits a variety of smooth muscles and nerves in vertebrates and may evoke vasoconstriction or vasodilatation depending on the vascular bed involved, the muscles resting tone and the dose given (Goodman & Gilman, 1980). It has a dual effect on the anterior byssus retractor muscle of Mytilus, potentiating both the contraction and relaxation of the sustained "catch" tension (Leake & Walker, 1980). It also decreases the contractile force of the leg muscle in the locust Schistocerca gregaria (Hill & Usherwood, 1961).

6.1.5 Acetylcholine

Acetylcholine, the acetic ester of choline is widely distributed in living organisms, being present in bacteria, fungi and plants as well as invertebrate and vertebrate animals (Leake & Walker, 1980).

Best known as a transmitter in animal nervous systems, it is typically synthesized in nerve axoplasm by the enzyme cholineacetyl transferase. Acetylcholine is then moved through the axoplasm to accumulate in vesicles in nerve endings. At synapses and neuromuscular junctions it is inactivated by the action of the enzyme acetylcholinesterase.

6.1.6 ɣ-Aminobutyric acid (GABA)

ɣ-Aminobutyric acid, a substance derived from glutamic acid, which occurs in high concentration in vertebrate brains and some invertebrate nervous systems, has been known for some time to have an inhibitory effect on a number of nervous preparations. It inhibits the activity of crayfish stretch receptors (Florey, 1954; Bazemore, Elliott & Florey, 1957) and has generalized inhibitory effects in crustacean (Kravitz, 1962) and insect (Ray, 1964) nervous systems. Leake & Walker (1980) have recently summarized GABA's normal actions in invertebrates as the inhibition of neuronal activity and the relaxation of muscles.

6.1.7 d.-Tubocurarine

d- Tubocurarine is one of a group of plant-derived substances used in South America as arrow poisons. It is a neuromuscular blocking agent that operates through preventing acetylcholine from depolarizing the post-synaptic membrane at the neuromuscular junction in skeletal muscles. It does not itself possess any acetylcholine-like activity at this site (Goodman & Gilman, 1980). d -Tubocurarine produces progressive neuro-muscular block leading to paralysis of skeletal muscle. Muscle paralysis can be produced in all vertebrate species, but there are variations in sensitivity from species to species and between different muscles in the same species (Goodman & Gilman, 1980).

6.1.8 Piperazine

Piperazine is used in the treatment of a number of intestinal nematode diseases. The expelled worms following the treatment, are described as narcotized, motionless and relaxed (Goodwin & Standen, 1954). Muscle tone and motility reappears upon immersion in physiological saline without piperazine. Its mode of action appears to be muscle paralysis by a reversible curare-like effect on neuromuscular junctions (Gutteridge, 1982).

6.1.9 Atropine

Atropine is an alkaloid from the plant Atropa belladonna. It acts by competing for the same receptors as acetylcholine, occupying them, and thus rendering applied acetylcholine ineffective. In general the peripheral effect of atropine are inhibitory. It also blocks the effect of injected cholinergic drugs, both peripherally and on the central nervous system in vertebrates (Laurence, 1973).

6.1.10 Pilocarpine

Pilocarpine is an alkaloid obtained from the leaves of the plant genus Pilocarpus. It possesses muscarinic action upon effector cells innervated by post-ganglionic autonomic cholinergic fibres. It does not possess significant nicotinic activity (Lewis, 1965).

6.1.11 Nicotine

Nicotine is an alkaloid obtained from the tobacco plant Nicotiana tabacum. It causes skeletal muscle to contract, having an acetylcholine-like effect causing depolarization and stimulation (Goodman & Gilman, 1980). This is the classic nicotinic action. It is short lived and develops into neuromuscular block where it can be shown to act in much the same way as d-tubocurarine (Goodman & Gilman, 1980).

6.1.12 Physostigmine (Eserine)

Physostigmine is one of a number of plant poisons extracted from the calabar bean Physostigma venenosum (see Goodman & Gilman, 1980). Its pharmacological effect is to antagonize the enzyme cholinesterase and prevent the destruction of acetylcholine. In this way it potentiates the effect of acetylcholine. It inhibits the enzyme by combining at the active centre or at a site spatially removed from the active centre called the peripheral anionic site. It forms an alternative substrate for the enzyme and is hydrolised extremely slowly (Goodman & Gilman, 1980).

6.1.13 Prostigmine (Neostigmine)

Prostigmine is a synthetic physostigmine-related compound. Evidence has been put forward to show that prostigmine not only possesses anticholinesterase activity but also acetylcholine properties (Lewis, 1965).

6.2 Materials and Methods

6.2.1 Cercarial drug exposure experiments

Recently emerged cercariae of T. patialense (see General materials and methods, Chapter 2.3) aged between 5 and 10 minutes were utilized in larval drug exposure experiments. Each basic experiment consisted of replicated groups of 10 such cercariae placed together in 3 mls of test solution in a 4.5 ml capacity square cross-section glass cuvette with optically flat walls. The cuvettes contained tap water in which drugs of known concentration

had been dissolved. The cuvettes were illuminated from above (approximately 3600 lux) and the cercariae within them observed under dark background conditions at $20^{o} \pm 2^{o}C$. The ability of cercariae to move was monitored by counting at five minute intervals the number of cercariae in each cuvette that were actively swimming immediately after a standardized mechanical stimulus produced by dropping a 11.1 g. ball bearing from a height of 5 cm onto the surface on which the experimental cuvettes were standing. In some experiments more or less frequent activity scorings were required.

6.2.2 Drugs utilized

In each group of replicated experiments on a particular drug, control cuvettes containing cercariae in tap water were always carried out in parallel to observe spontaneous levels of activity in the absence of drugs. The following drugs were utilized in cercarial drug exposure experiments.

Adrenaline (Richter). Concentration range between 2,000-31.25 ppm (w/v): i.e. (5.46mM-0.17mM).

Noradrenaline (Arterenol)(Sigma). Concentration range between 500-15.63 ppm (w/v): i.e. (2.43mM-0.08mM).

Dopamine (sigma). Concentration range between 2,000-31.25 ppm (w/v): i.e. (10.54mM-0.17mM).

5-Hydroxytryptamine (Serotonin) (5HT) (Sigma): Concentration range between 2,000-31.25 ppm (w/v): i.e. (5.16mM-0.08mM).

Acetylcholine chloride (Sigma). Concentration range between 2,000-31.25 ppm (w/v): i.e. (2.95mM-0.05mM).

γ-Aminobutyric acid (GABA)(Sigma). Concentration range between 8,000-500 ppm (w/v): i.e. (77.59mM-4.85mM).

d-Tubocurarine chloride Hydrate (Sigma). Concentration range between 2,000-31.25 ppm (w/v): i.e. (2.87mM-0.05mM).

Piperazine citrate (Sigma). Concentration range between 8,000-125 ppm (w/v): i.e. (37.34mM-1.17mM).

Atropine sulphate (Sigma). Concentration range between 2,000-31.25 ppm (w/v): i.e. (5.76 mM-0.09mM).

Pilocarpine HCI (Sigma). Concentration range between 2,000-31.25 ppm (w/v): i.e. (8.17mM-0.13mM).

Nicotine (Sigma). Concentration range between 400-20 ppm (w/v): i.e. (2.46mM-0.12mM).

Physostigmine (Eserine) sulfate (Sigma). Concentration range between 2,000-15.63 ppm (w/v): i.e. (6.15mM-0.05mM).
Prostigmine (Neostigmine) bromide (Sigma). Concentration range between 2,000-31.25 ppm (w/v): i.e. (6.59mM-0.10mM).

In all cases a stock solution was made up at the highest concentration and then lower concentrations produced by appropriate serial dilutions.

6.2.3 Decaudation experiments

In order to study the effects of drugs on separated cercarial heads and tails and to investigate the spontaneous activity of these regions of the larvae some drug exposure experiments were performed on decaudated preparations. For separated heads and tails any muscular movement was regarded as positive activity.

To separate cercarial heads from tails, cercariae were cooled to 2°C for about 20 minutes in order to reduce their motility. They were then placed in 5 mls of 2°C tap water in a hypodermic syringe and expelled through a 40 mm 11/10 size needle (Gillette). This technique resulted in the separation of nearly all heads from tails which were then collected separately and left for five minutes to warm up to room temperature before being used in subsequent experiments. The following drugs and concentrations were utilized in these experiments;
Physostigmine (Eserine) sulfate (0.19mM); atropine sulfate (5.76mM); nicotine (1.23mM) and adrenaline (1.37mM).

6.2.4 Drug antagonists

Possible antagonists of some of the drugs were tested in variants of the standard exposure experiments. The effects of such potential antagonists were studied in two different ways:
(i) By removing 1.5 ml of the drug solution and replacing it with 1.5 ml of a solution containing the same concentration of the drug and twice that concentration of the antagonist.
(ii) By removing the drug solution and rinsing the cercariae with tap water twice and then adding 3 mls of the antagonist solution.

The potential antagonists used were:

1) For physostigmine (eserine) (0.19mM): acetylcholine chloride (11.01mM and 5.50mM)
2) For nicotine (1.23mM): d-tubocurarine chloride (1.44mM)

3) For piperazine citrate (4.65mM): acetylcholine chloride (11.01mM).

6.3 Results

6.3.1 Drug effects on entire T. patialense cercariae

The pattern of inhibition of elicited swimming activity by T. patialense cercariae as a result of exposure to differing concentrations of a variety of drugs and for periods up to four hours after emergence are illustrated graphically in Figures 6.1 to 6.13 inclusive.

The drug-specific patterns represented in the different figures are as follows: adrenaline (Figure 6.1); noradrenaline (Figure 6.2); dopamine (Figure 6.3); 5-Hydroxytryptamine (Figure 6.4); acetylcholine (Figure 6.5); GABA (Figure 6.6); d-tubocurarine (Figure 6.7): piperazine (Figure 6.8); atropine (Figure 6.9); pilocarpine (Figure 6.10); nicotine (Figure 6.11); physostigmine (Figure 6.12); prostigmine (Figure 6.13).

In some cases these figures have been used to provide estimates of the time to 50% inhibition of elicited activity (T_{50}) for different drug concentrations. The relationship of T_{50} to drug concentration of particular drugs are shown in Figures 6.14 to 6.17).

It is convenient to describe the drug effects on intact cercariae in terms of six functionally distinct drug types i.e. catecholamines, indolealkylamines, acetylcholine, γ-aminobutyric acid, postsynaptic cholinergic receptor blockers and acetylcholinesterase inhibitors.

6.3.1.1 Catecholamines

Cerecariae were exposed to the three different common catecholamines at the following concentration levels: adrenaline;5.46,2.73,1.37, 0.69, 0.34 and 0.17mM; noradrenaline; 2.43,1.22,0.61,0.30,0.15 and 0.08mM: dopamine; 10.55,5.27,2.64,1.32,0.66,0.33 and 0.17mM. The results recorded in Figures 6.1, 6.2 and 6.3 show that each of these catecholamines exhibits some ability to inhibit the elicited swimming activity of T. patialense cercariae. When relative inhibitory effects are examined using the relationships between T_{50} for each drug and drug millimolarity (see Figure 6.14) it is clear that adrenaline has a greater dose-related inhibitory effect

Figure 6.1 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of adrenaline at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

(The tap water controls for the experimental replicates illustrated in Figures 6.1-6.13 never showed any reduction in the proportion of cercariae demonstrating elicited swimming below 1.0 up to 185 minutes after the beginning of any experiment. Between 185 and 240 minutes the overall level of swimming activity in control cuvettes never dropped below 0.93. In view of these high and consistent levels of activity in control cuvettes, T_{50} estimates for Figures 6.14-6.18 have been obtained assuming that all controls show a value of 1.0.)

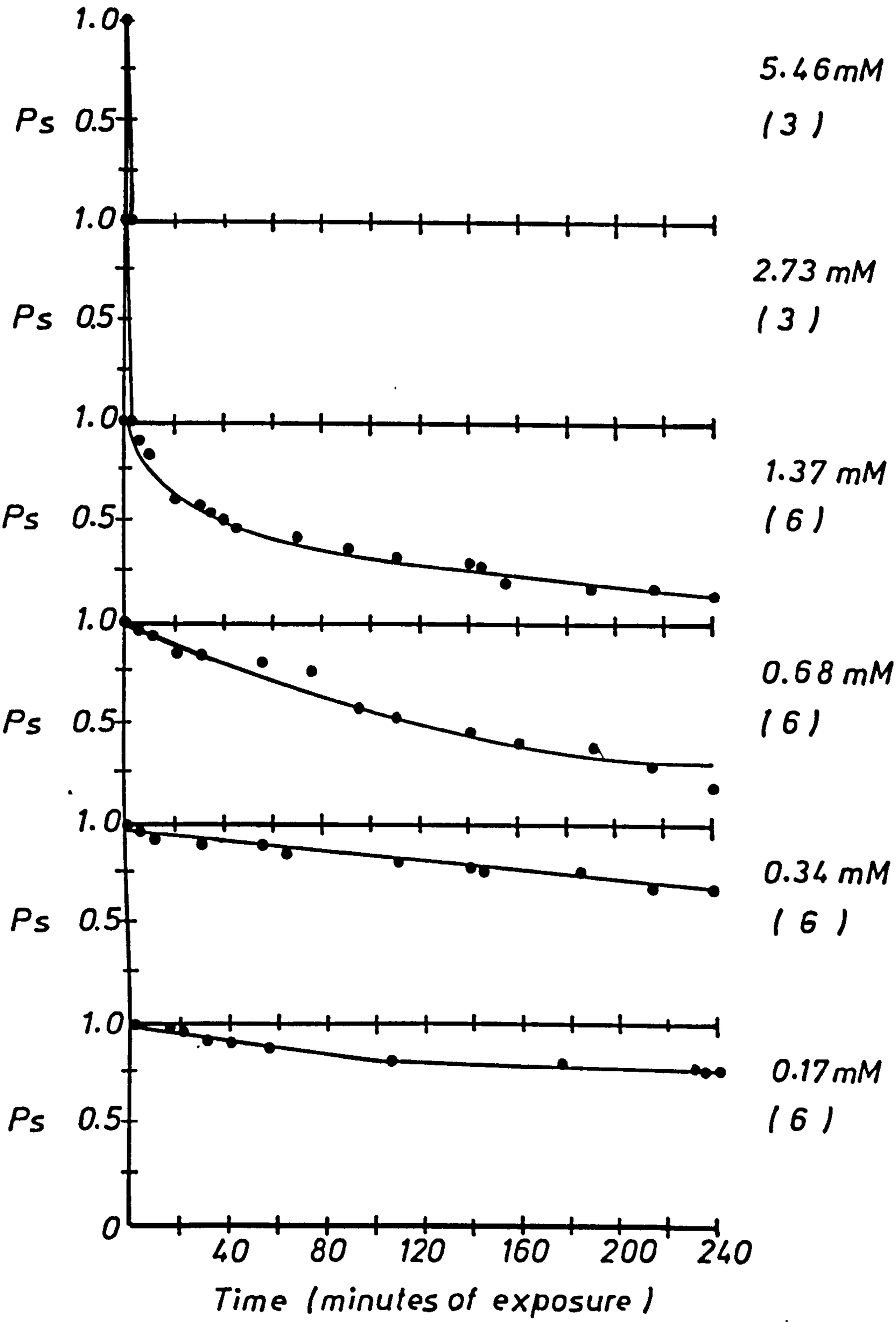

1.0
Ps 0.5
5.46mM
(3)
2.73 mM
(3)
1.37 mM
(6)
0.68 mM
(6)
0.34 mM
(6)
0.17mM
(6)
0
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.2 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of noradrenaline at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

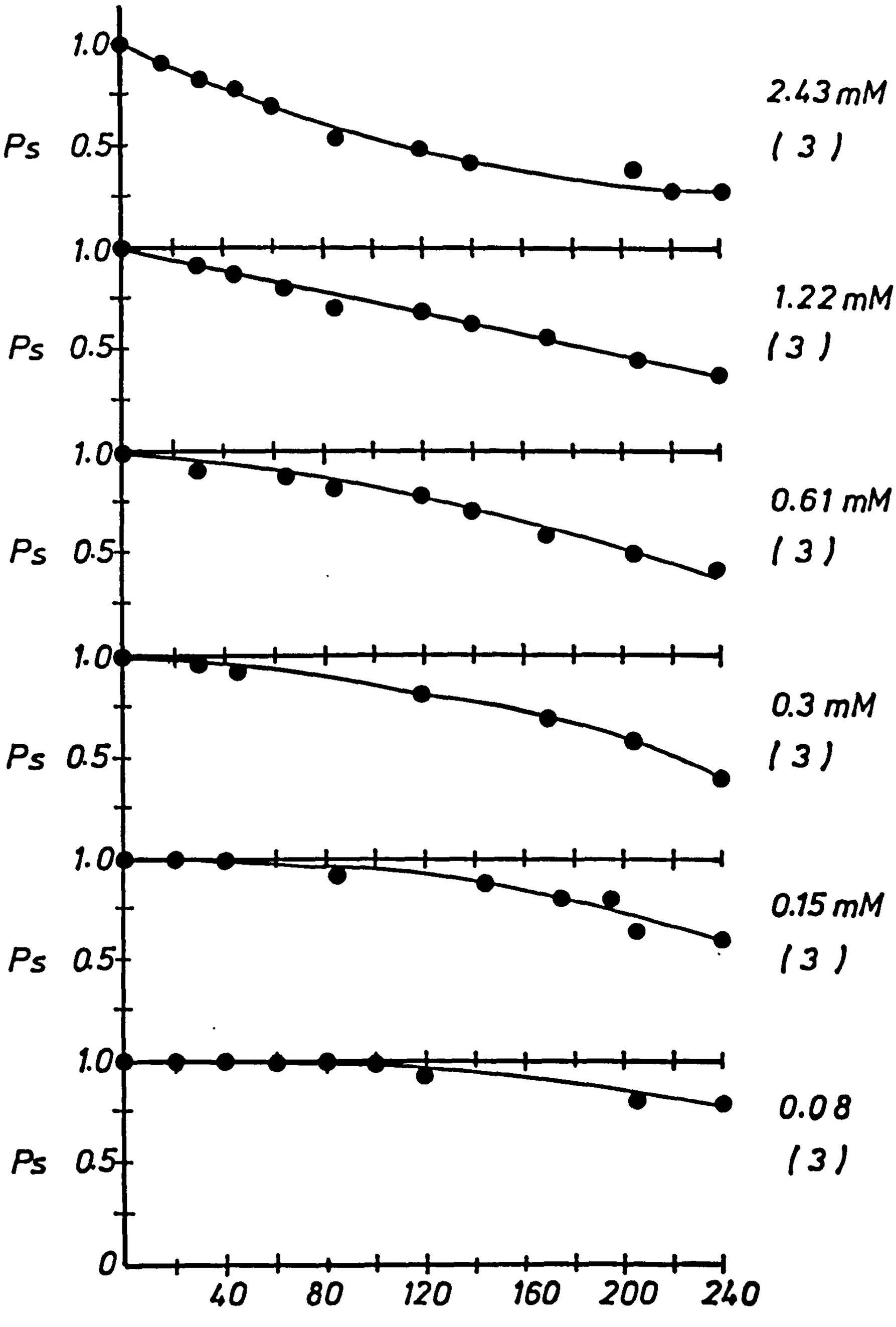
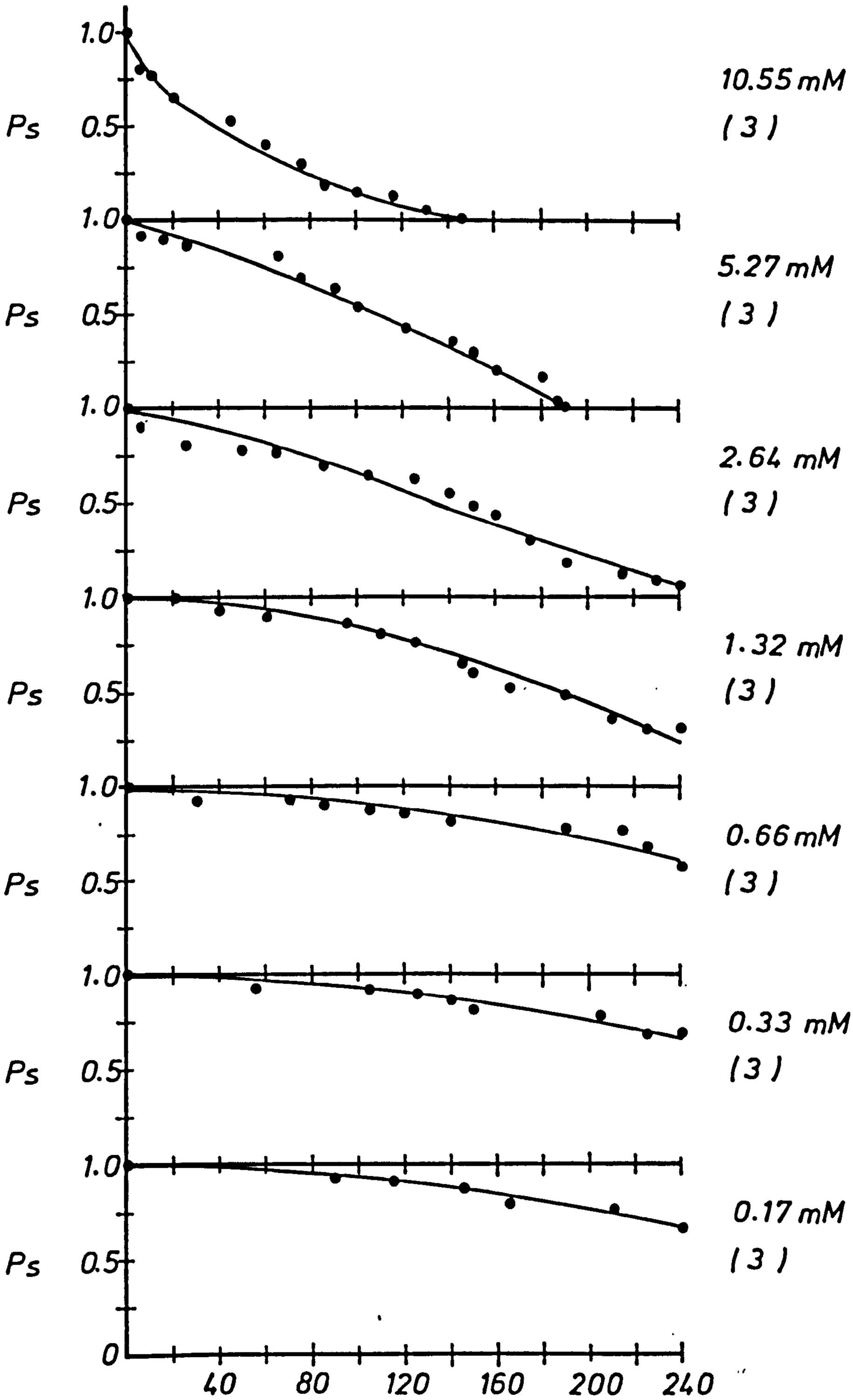

1.0
Ps 0.5
2.43 mM
(3)
1.22 mM
(3)
0.61 mM
(3)
0.3 mM
(3)
0.15 mM
(3)
0.08
(3)
0
40 80 120 160 200 240
Time (minutes of exposure)

Figure 6.3 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of dopamine at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.


1.0
Ps
0.5
10.55 mM
(3)
1.0
5.27 mM
(3)
Ps
0.5
1.0
2.64 mM
(3)
Ps
0.5
1.0
1.32 mM
(3)
Ps
0.5
1.0
0.66 mM
(3)
Ps
0.5
1.0
0.33 mM
(3)
Ps
0.5
1.0
0.17 mM
(3)
Ps
0.5
0
40
80
120
160
200
240
Time (minutes of exposure)

than noradrenaline or dopamine which have similar dose-related efficacy.

Inspection of Figures 6.1-3 reveals that for all three catecholamines, decreasing drug concentration diminishes levels of inhibition through time in a continuous fashion. In the case of adrenaline , external drug concentrations above 2.5mM are extremely effective in eliminating elicited cercarial swimming behaviour. At these concentrations, all tested larvae are immobile within 60 seconds of exposure to the drug.

6.3.1.2 Indolealkylamines

Only one indolealkylamine was tested against elicited T. patialense cercarial swimming activity. 5HT was utilized at the following millimolarities (mM): 5.16,2.58,1.29,0.65, 0.32,0.16,0.08. Within the four hour period of the drug exposure experiments (see Figures 6.4,6.15) only drug concentrationsabove 1.2mM were able to induce a 50% reduction in swimming activity. Figure 6.4 also shows that the inhibitory effect of 5-Hydroxytryptamine through time were declined in a continuous fashion with decreasing drug concentration.

6.3.1.3 Acetylcholine

Acetylcholine at concentrations of 11.0,5.50,2.75,1.38,0.69, 0.34,0.17mM was used in cercarial exposure experiments. Figure 6.5 reveals that this transmitter shows some apparent ability to inhibit elicited swimming activity. At 11mM for instance, all activities had ceased after 200 minutes.

Figure 6.17 shows the relationship between estimated T_{50} for this drug and drug concentration.

6.3.1.4 γ-Aminobutyric acid (GABA)

GABA was utilized in drug exposure experiments at concentrations of 77.60,38.80,19.40,9.70 and 4.85mM. Even at the highest concentration utilized (77.60mM) it was difficult to demonstrate anything other than the most minimal degree of swimming inhibition. Inhibition never reached the 50% level at any GABA concentration utilized during four-hour duration experiments (see Figure 6.6).

Figure 6.4 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of 5-hydroxytryptamine (serotonin) at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

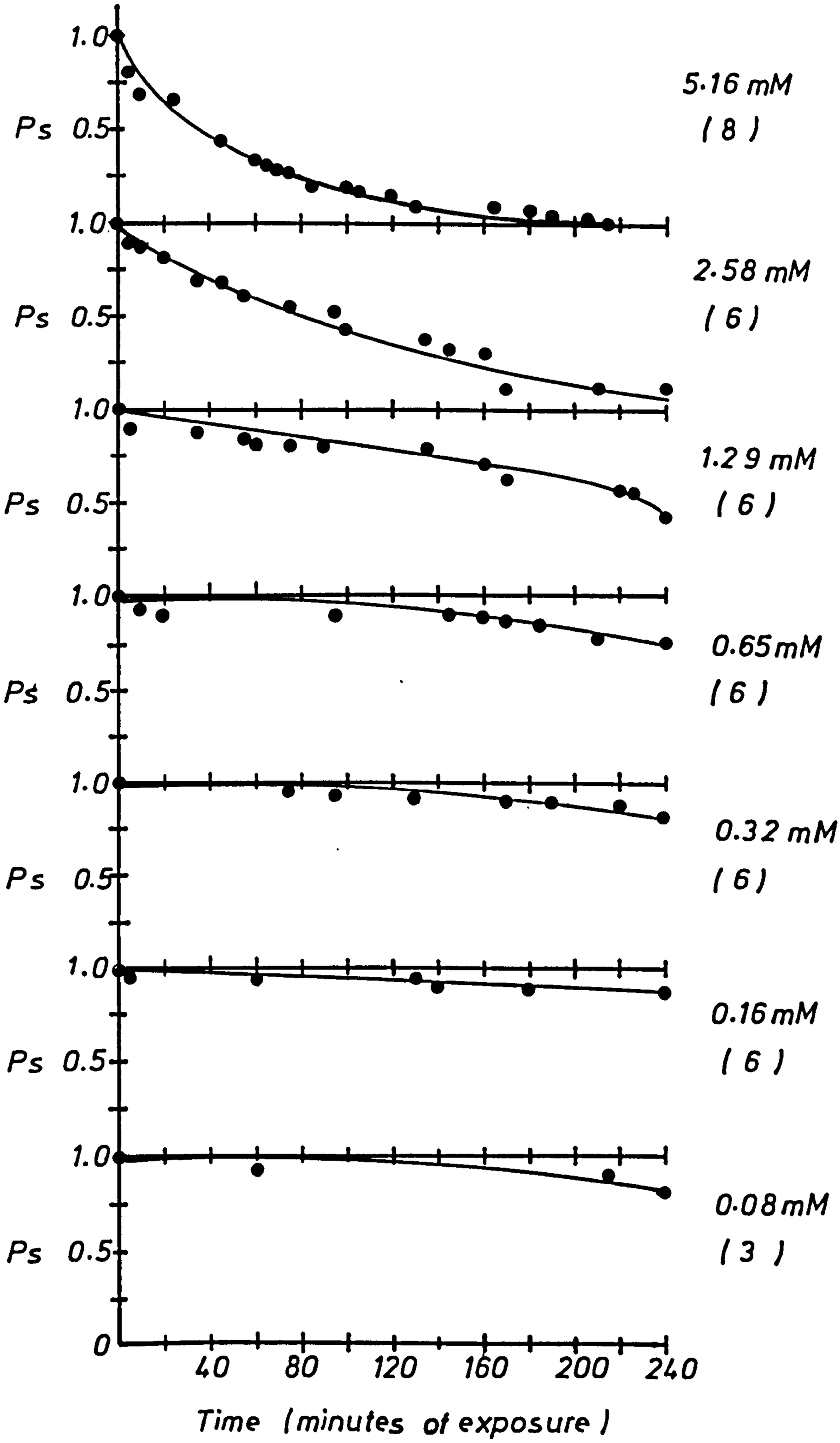

1.0
Ps 0.5
5.16 mM
(8)
2.58 mM
(6)
1.29 mM
(6)
0.65mM
(6)
0.32 mM
(6)
0.16mM
(6)
0.08mM
(3)
0
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.5 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of acetylcholine at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

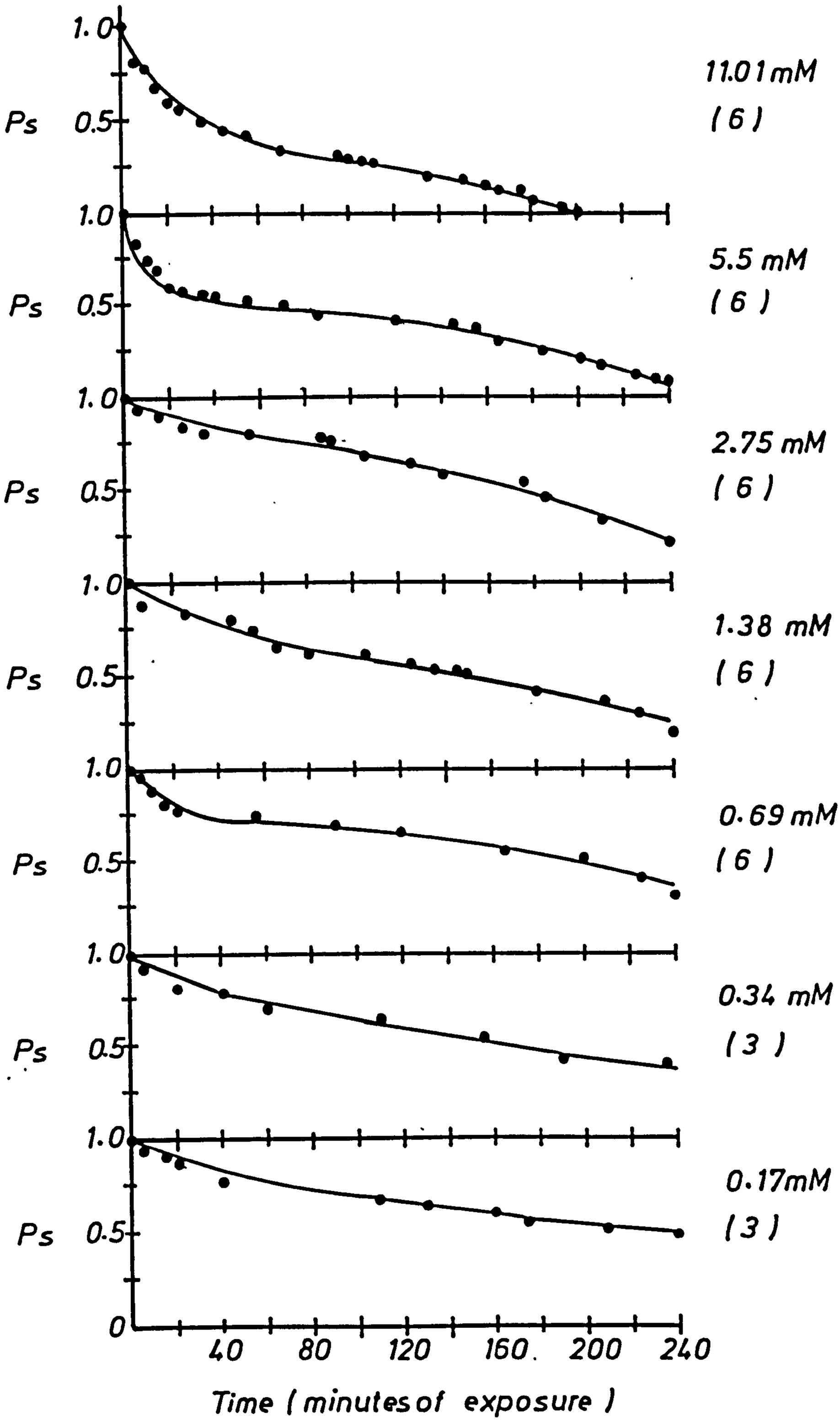
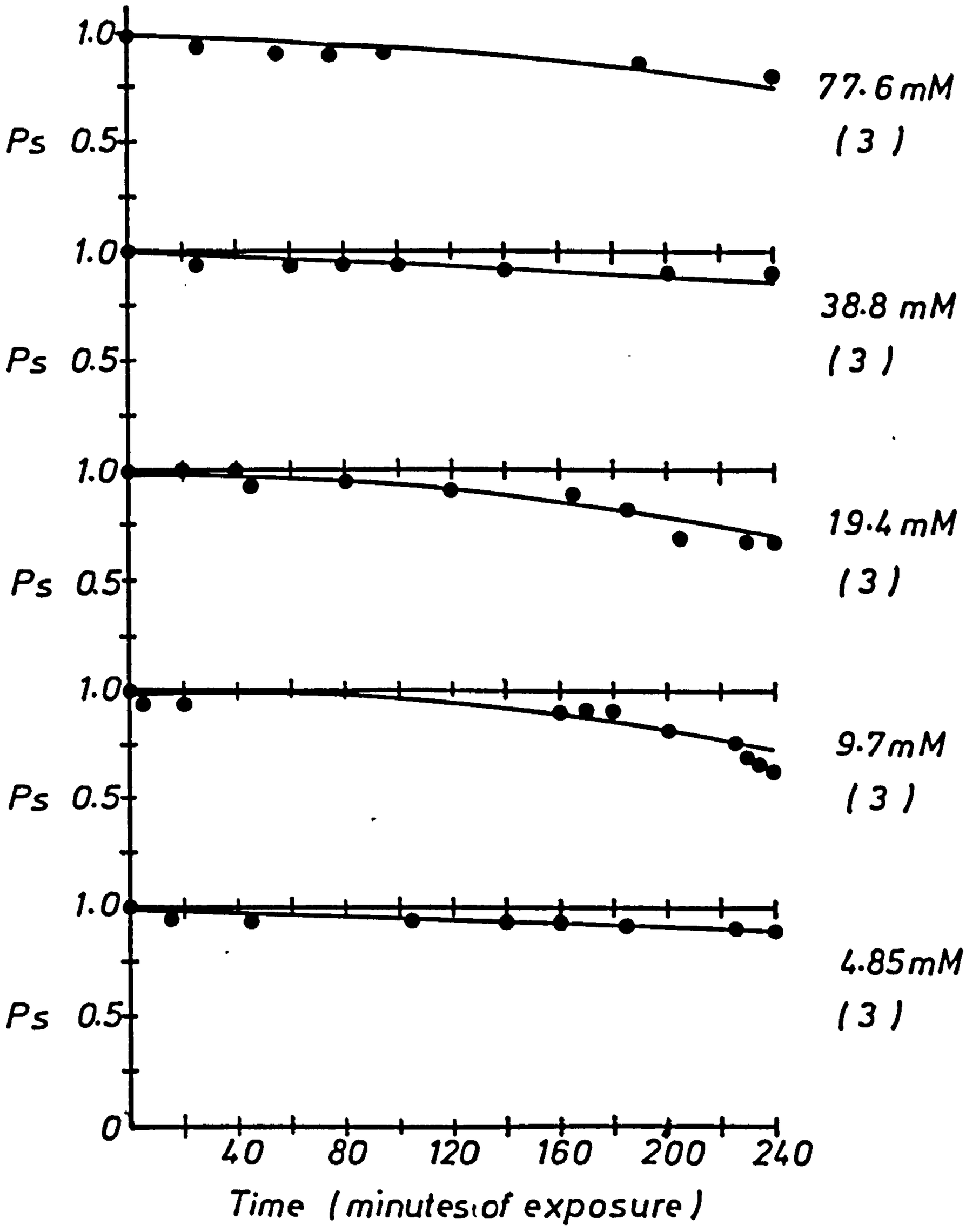

1.0
0.5
Ps
11.01 mM
(6)
5.5 mM
(6)
2.75 mM
(6)
1.38 mM
(6)
0.69 mM
(6)
0.34 mM
(3)
0.17mM
(3)
0
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.6 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of γ-aminobutyric acid at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.


1.0
Ps 0.5
0
77.6 mM
(3)
38.8 mM
(3)
19.4 mM
(3)
9.7mM
(3)
4.85mM
(3)
40
80
120
160
200
240
Time (minutes of exposure)

6.3.1.5 Post-synaptic cholinergic receptor blockers

Five drugs with a proven or suggested ability to block cholinergic receptor sites were utilized in this study at the following concentrations: d-tubocurarine; 2.88,1.44,0.72,0.34,0.18,0.09 and 0.05mM: piperazine;37.34,18.67,9.33,4.65,6.34 and 1.17mM: atropine, 5.76,2.88,1.44,0.72,0.36,0.18 and 0.09mM: pilocarpine, 8.17,4.09,2.04,1.02,0.51,0.26 and 0.13mM; nicotine, 2.47,1.23, 0.62,0.41 and 0.12mM. The relationships of P_s against time at different drug concentrations for these five drugs are illustrated in Figures 6.7-6.11 respectively. All showed some ability to reduce elicited cercarial swimming, and in all five cases one or more of the concentrations utilized produced a 50% inhibition within the four hour period of the experiments.

The five drugs can be categorized into three groups on the basis of their inhibitory behaviour (see Figure 6.16).Pilocarpine, piperazine and d-tubocurarine produce low, dose-related inhibitory effects of similar magnitude when the drugs are compared on a similar millimolar basis. Atropine shows similar but considerably more potent inhibitory effect than that of the inhibitions in the first group. Figure 6.16, for instance, reveals that at all parts of the investigated range of drug effects piperazine concentrations (mM) some 20 to 30 times greater than those of atropine were required to produce equivalent T_{50} responses.

The third type of response is that associated with nicotine. Figure 6.11 shows that the P_s against time relationships at different drug concentrations show a complex form. At 2.47mM P_s drops rapidly and progressively with time until after 10 minutes of exposure no cercariae are mobile. At all lower concentrations, however, the pattern is more complicated. After very rapid initial declines in elicited cercarial swimming activity a subsequent reversal of part of this inhibition occurs. From between about 20 minutes to 60 minutes into the exposure period at different concentrations the level of activity begins to slowly decrease once again. The form of these time and dose-dependent relationships mean that it is impossible to estimate T_{50} values in the same fashion that has been employed for most of the other drugs which have been used.

Figure 6.7 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of d-tubocurarine at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

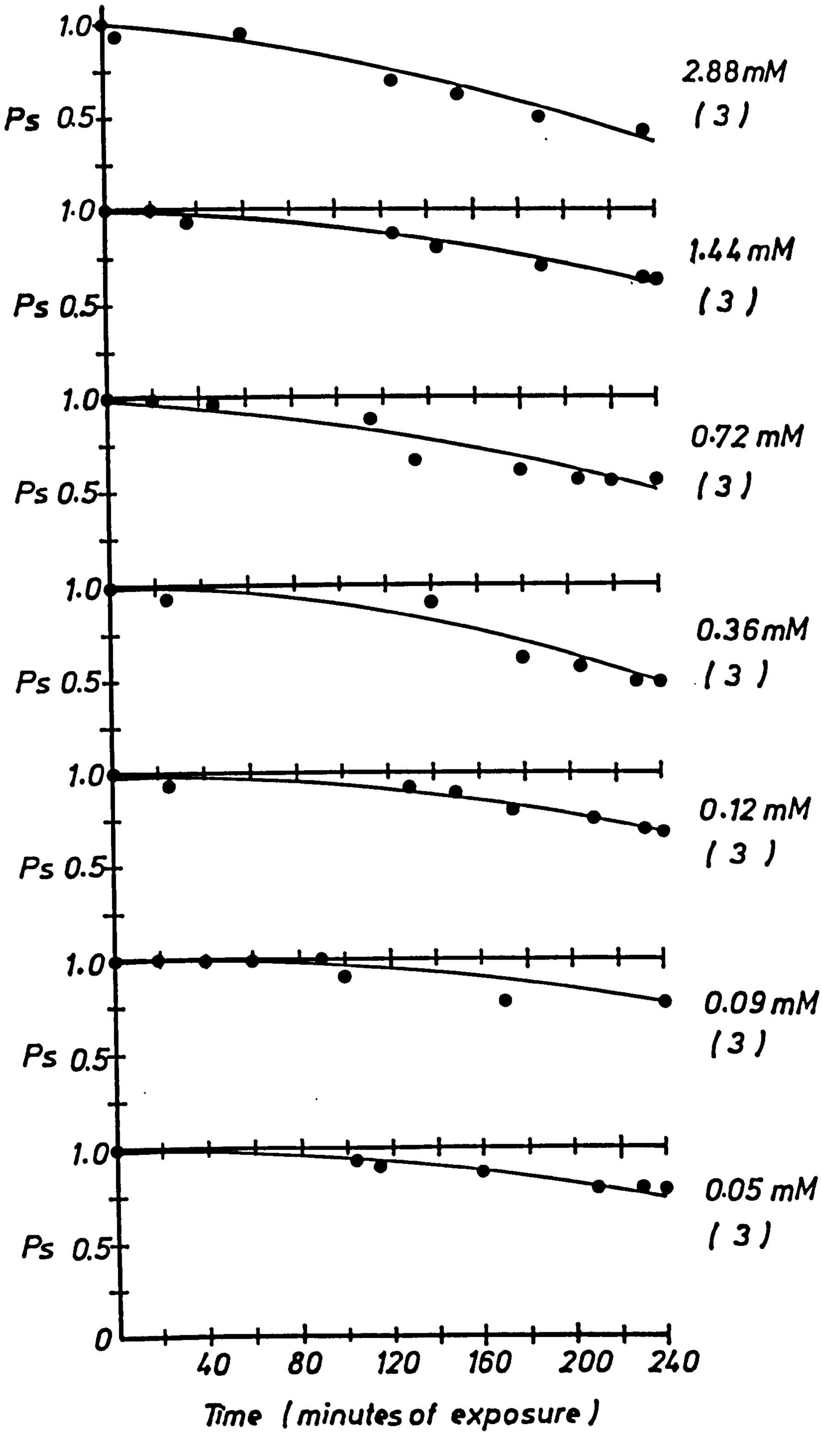

1.0
Ps 0.5
2.88mM
(3)
1.44mM
(3)
0.72 mM
(3)
0.36mM
(3)
0.12 mM
(3)
0.09mM
(3)
0.05 mM
(3)
0
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.8 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of piperazine at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten replicates. The curves were fitted by eye.

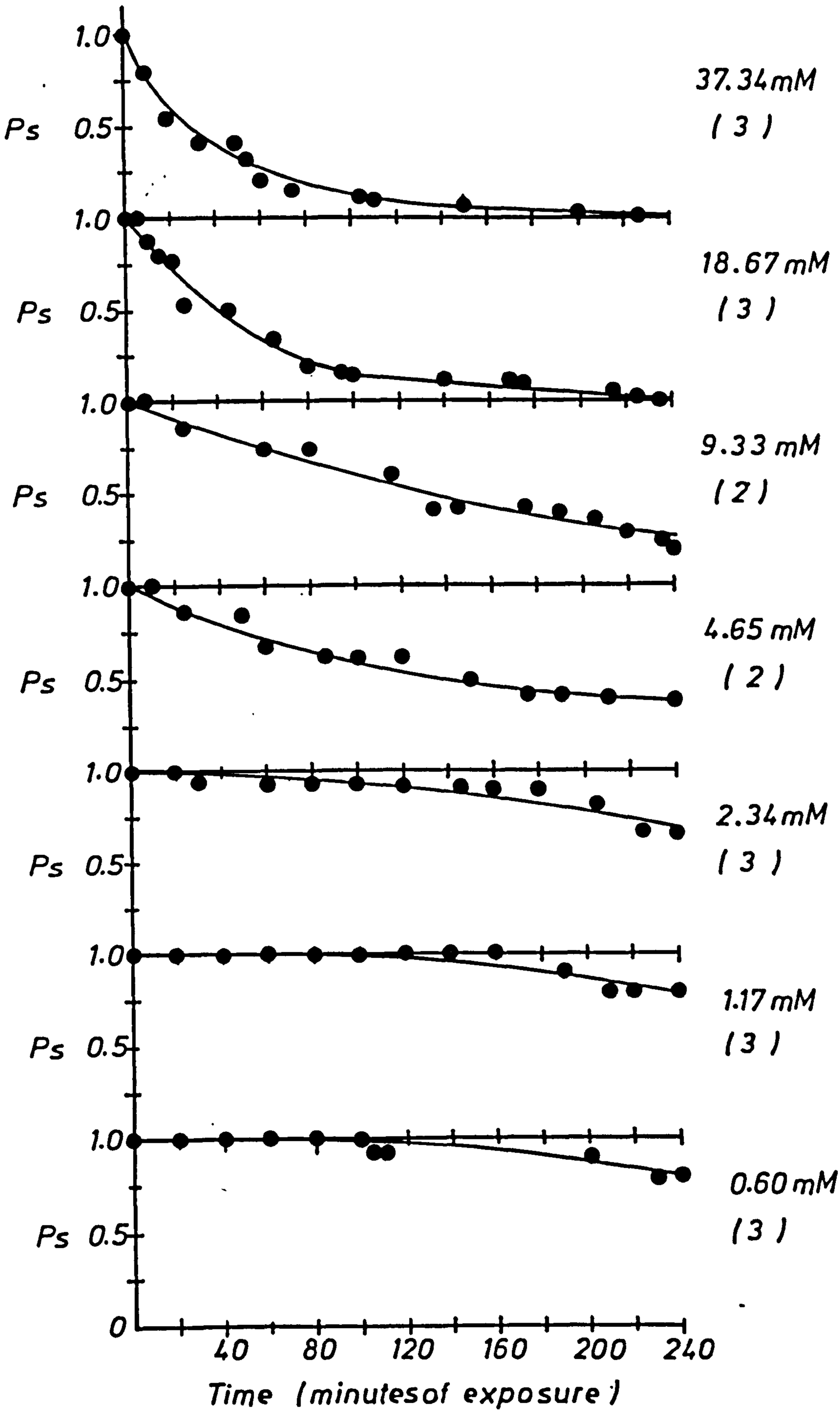

1.0
Ps 0.5
37.34mM
(3)
18.67 mM
(3)
9.33 mM
(2)
4.65 mM
(2)
2.34mM
(3)
1.17 mM
(3)
0.60 mM
(3)
0
40
80
120
160
200
240
Time (minutesof exposure)

Figure 6.9 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of atropine at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

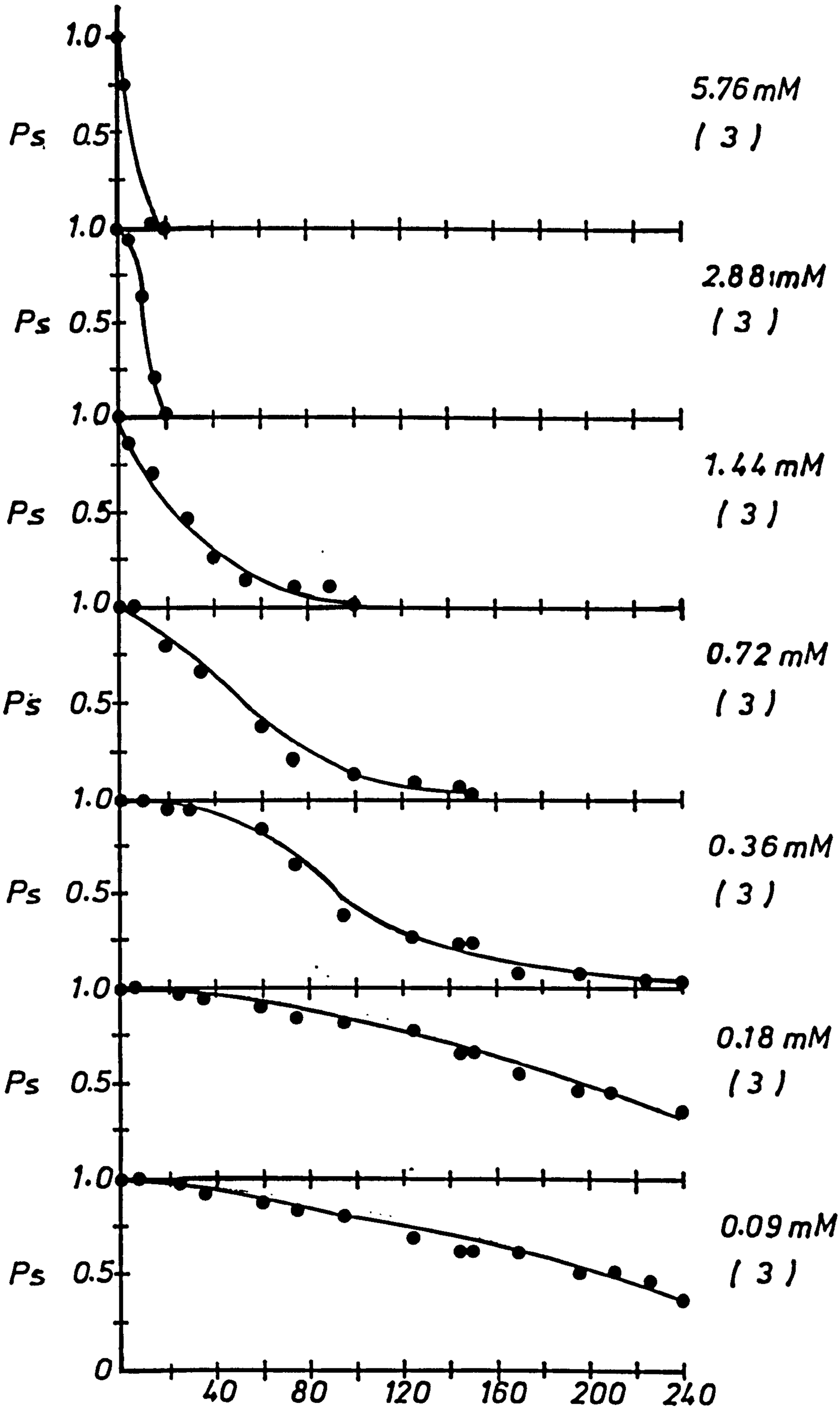

1.0
Ps 0.5
1.0
5.76 mM
(3)
Ps 0.5
1.0
2.88 mM
(3)
1.0
Ps 0.5
1.0
1.44 mM
(3)
Ps 0.5
1.0
0.72 mM
(3)
Ps 0.5
1.0
0.36 mM
(3)
Ps 0.5
1.0
0.18 mM
(3)
Ps 0.5
1.0
0.09 mM
(3)
Ps 0.5
0
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.10 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of pilocarpine at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

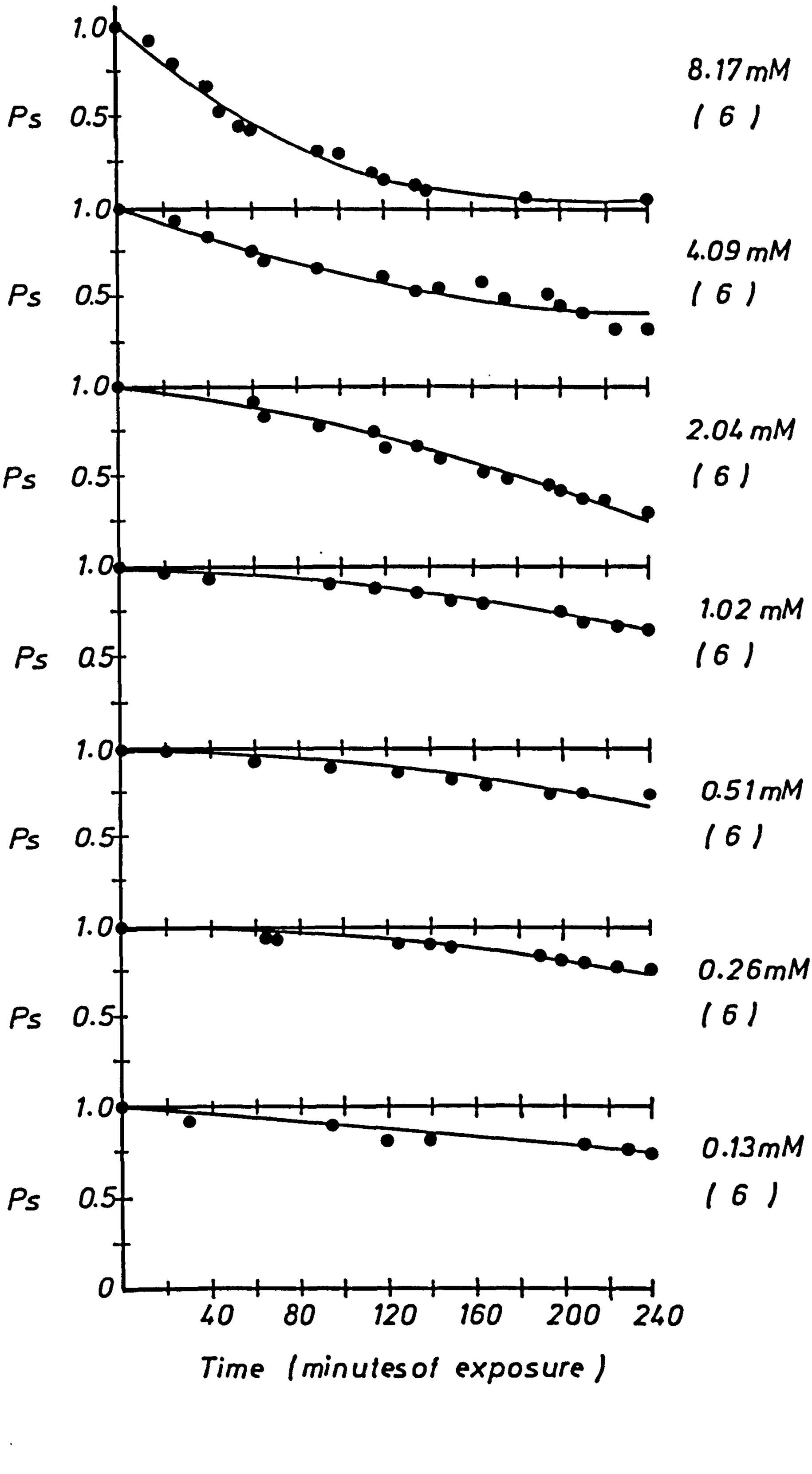
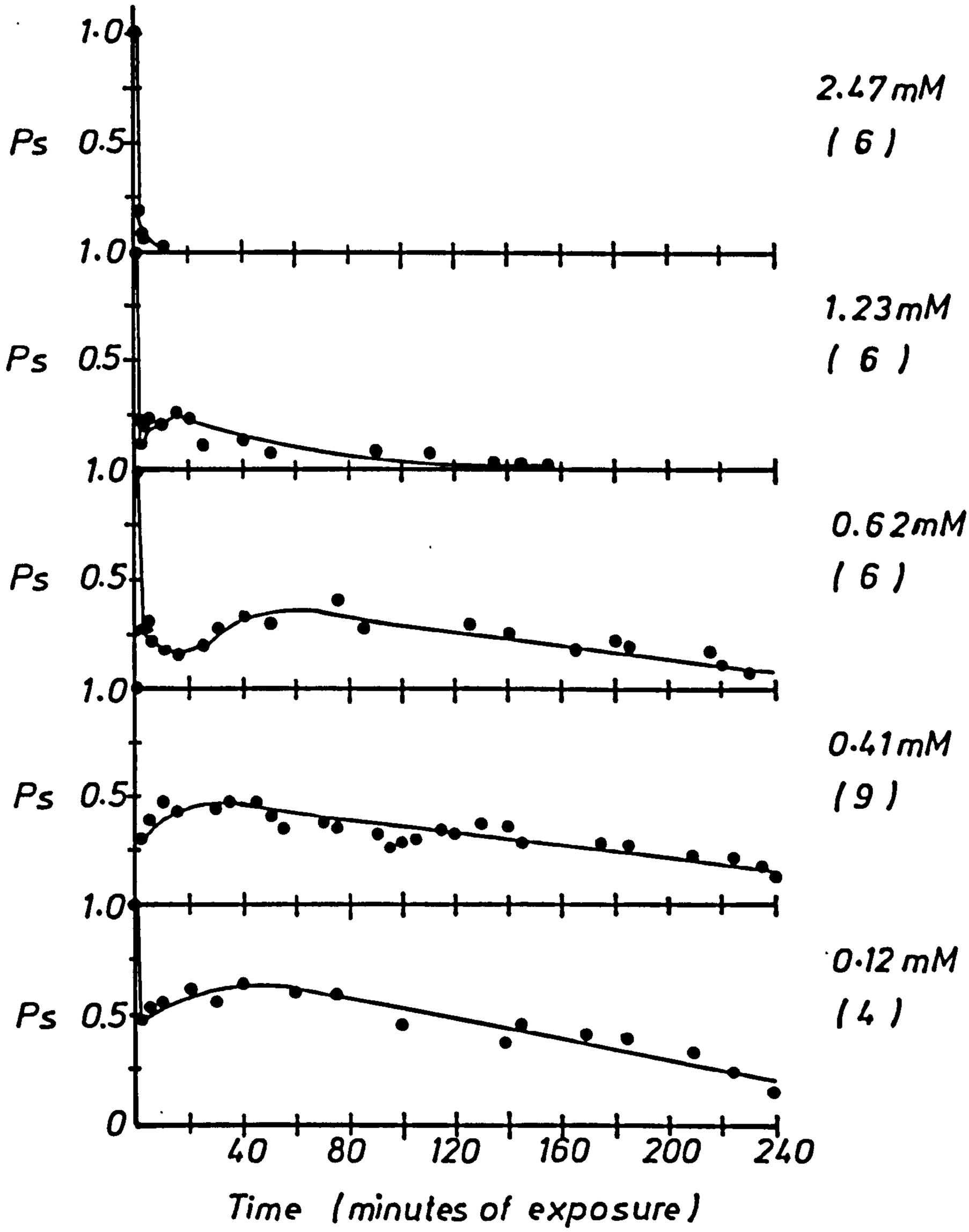

1.0
Ps 0.5
8.17 mM
(6)
1.0
Ps 0.5
4.09 mM
(6)
1.0
Ps 0.5
2.04 mM
(6)
1.0
Ps 0.5
1.02 mM
(6)
1.0
Ps 0.5
0.51mM
(6)
1.0
Ps 0.5
0.26mM
(6)
1.0
Ps 0.5
0.13mM
(6)
0
40 80 120 160 200 240
Time (minutesof exposure)

Figure 6.11 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of nicotine at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.


Ps
1.0
0.5
2.47mM
(6)
1.23mM
(6)
0.62mM
(6)
0.41mM
(9)
0.12 mM
(4)
0
40
80
120
160
200
240
Time (minutes of exposure)

6.3.1.6 Acetylcholinesterase inhibitors

Two acetylcholinesterase inhibitors were utilized in drug exposure experiments at the following concentrations namely physostigmine (eserine); 6.15,3.07,1.54,0.77,0.38,0.19,0.10 and 0.05mM; prostigmine (neostigmine); 6.60,3.30,1.65,0.83,0.41,0.21 and 0.10mM. Both were able to produce dose-related inhibitions of elicited swimming behaviour of considerable magnitude (see Figures 6.12,6.13 and 6.17). The P_s against time responses of eserine, however, were much more complex than those of neostigmine. In particular, the behaviour of the former drug was rather different in its time-dependence at different concentrations. Above 0.77mM activity very rapidly dropped to zero and the inhibition was irreversible in the presence of the drug. At concentrations below 0.77mM there was a partial reversal of initial high levels of inhibition between about five and 60 minutes of drug exposure. This complexity made it difficult to provide clear estimates of T_{50} for this drug at concentrations between 3.00 and 0.02mM. Despite this difficulty, it is obvious that throughout this range of concentrations physostigmine is able to produce an inhibition of swimming in excess of 50% within five minutes of the beginning of the exposure period. This response means that eserine has the most powerful effect of any of the drugs used in this study.

6.3.2 Drug effects on separated heads and tails

As shown in Figure 6.18 the effect of adrenaline,when compared with tap water controls of both parts of the larvae, is very much more marked on the activity of the head than of the tail. Tail activity seems to be completely unaffected by the presence of 1.37mM adrenaline whereas head activity was reduced by 50% with respect to controls within 60 minutes. Figure 6.18 also demonstrates a dramatic head-tail difference in the effect of 0.19mM physostigmine. Heads in this drug cease all muscular activity within one minute of exposure. Tails in contrast, compared with tail tap water controls, show a gradual increase in inhibition up to about 30 minutes after exposure but thereafter this maximal level of inhibition (i.e. about 70%) is rapidly reversed. Figure 6.19 illustrates the effect of atropine and nicotine on separated heads and tails. 5.76mM atropine produces a smooth decline in muscular activity in both heads and tails

Figure 6.12 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of physostigmine (eserine) at varying times after the initiation of drug expesure.

The number between brackets (n) represents the number of replicates of ten cercariae.

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of ten cercariae replicates. The curves were fitted by eye.

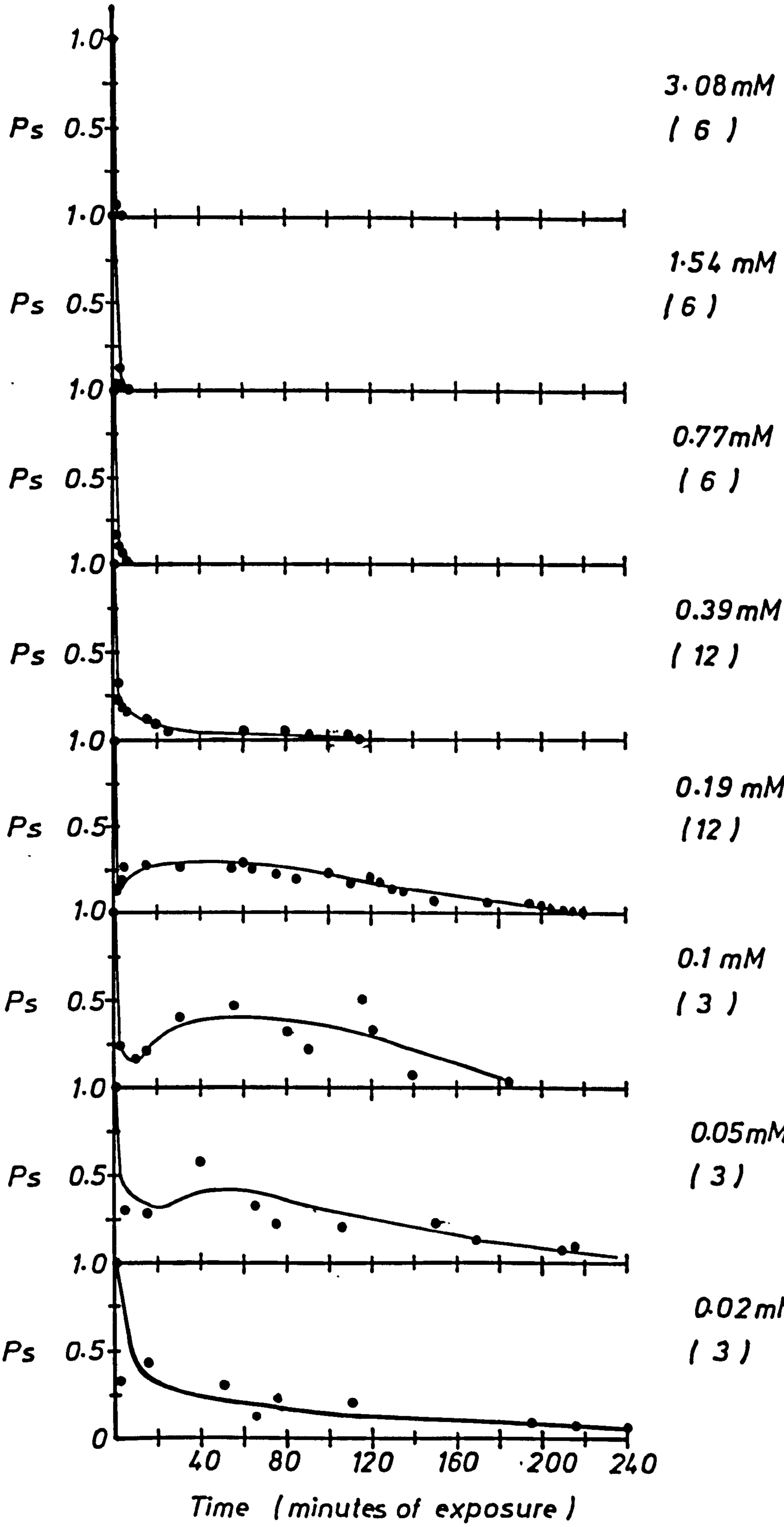

1.0
Ps 0.5
1.0
3·08 mM
(6)
Ps 0.5
1.0
1·54 mM
(6)
Ps 0.5
1.0
0.77mM
(6)
Ps 0.5
1.0
0.39mM
(12)
Ps 0.5
1.0
0.19 mM
(12)
Ps 0.5
1.0
0.1 mM
(3)
Ps 0.5
1.0
0.05mM
(3)
Ps 0.5
0
0.02mM
(3)
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.13 The proportion of cercariae (Ps) showing elicited swimming behaviour in different concentrations of prostigmine (neostigmine) at varying times after the initiation of drug exposure.

The number between brackets (n) represents the number of replicates of ten cercariae

Each data point (.) represents the mean proportional level of elicited swimming activity in the stated number of cercariae replicates. The curves were fitted by eye.

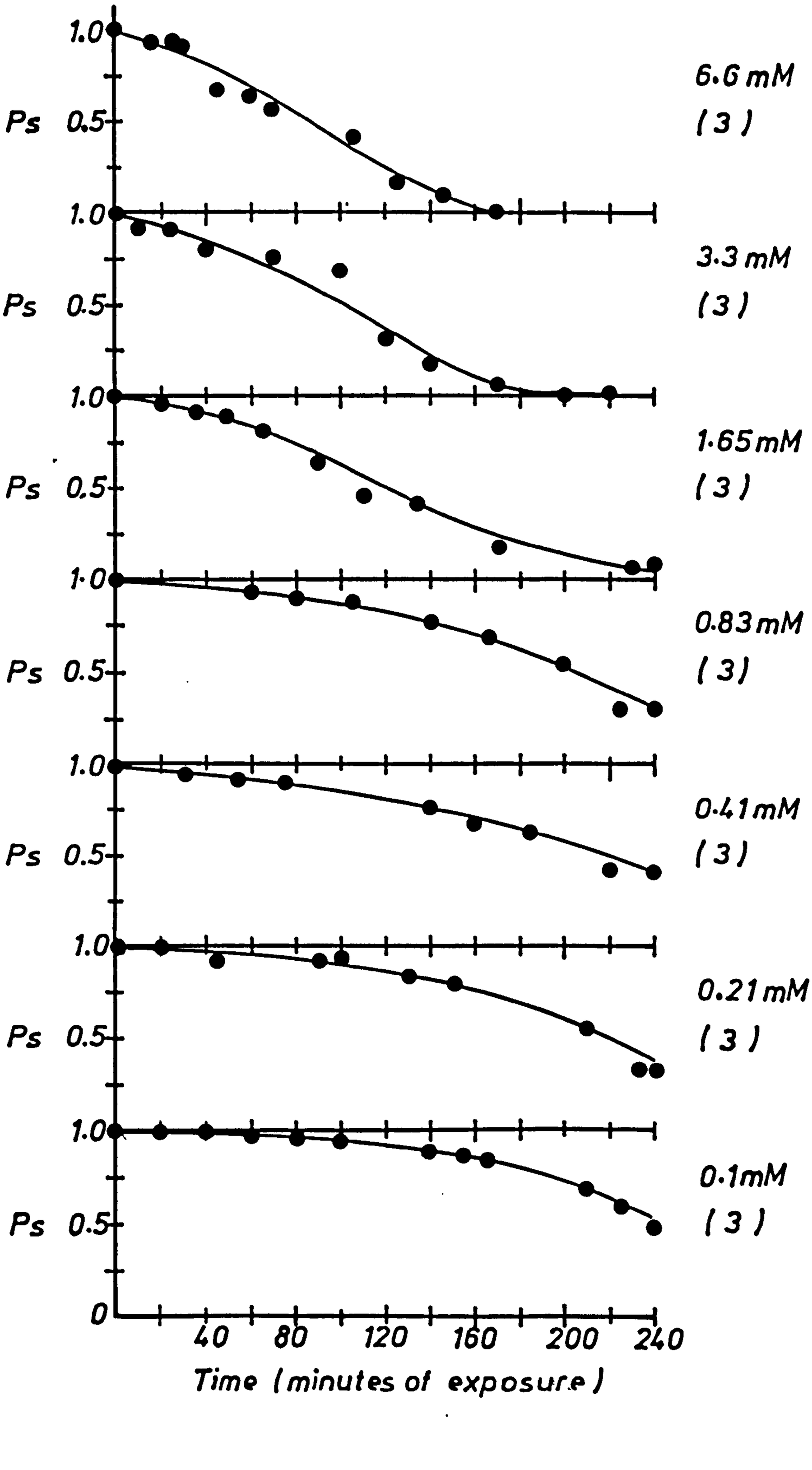

1.0
Ps 0.5
6.6 mM
(3)
3.3 mM
(3)
1.65mM
(3)
0.83mM
(3)
0.41mM
(3)
0.21mM
(3)
0.1mM
(3)
0
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.14 The relationship of estimated times to 50% inhibition of elicited swimming activity (T_{50}) against drug concentration (mM) for the catecholamines, adrenaline, noradrenaline and dopamine.

● represents adrenaline

♦ represents noradrenaline

■ represents dopamine

(The T_{50} estimates were obtained graphically from Figures 6.1, 6.2 and 6.3 with the assumptions concerning activity levels in control cuvettes described in detail in the legend to Figure 6.1.)

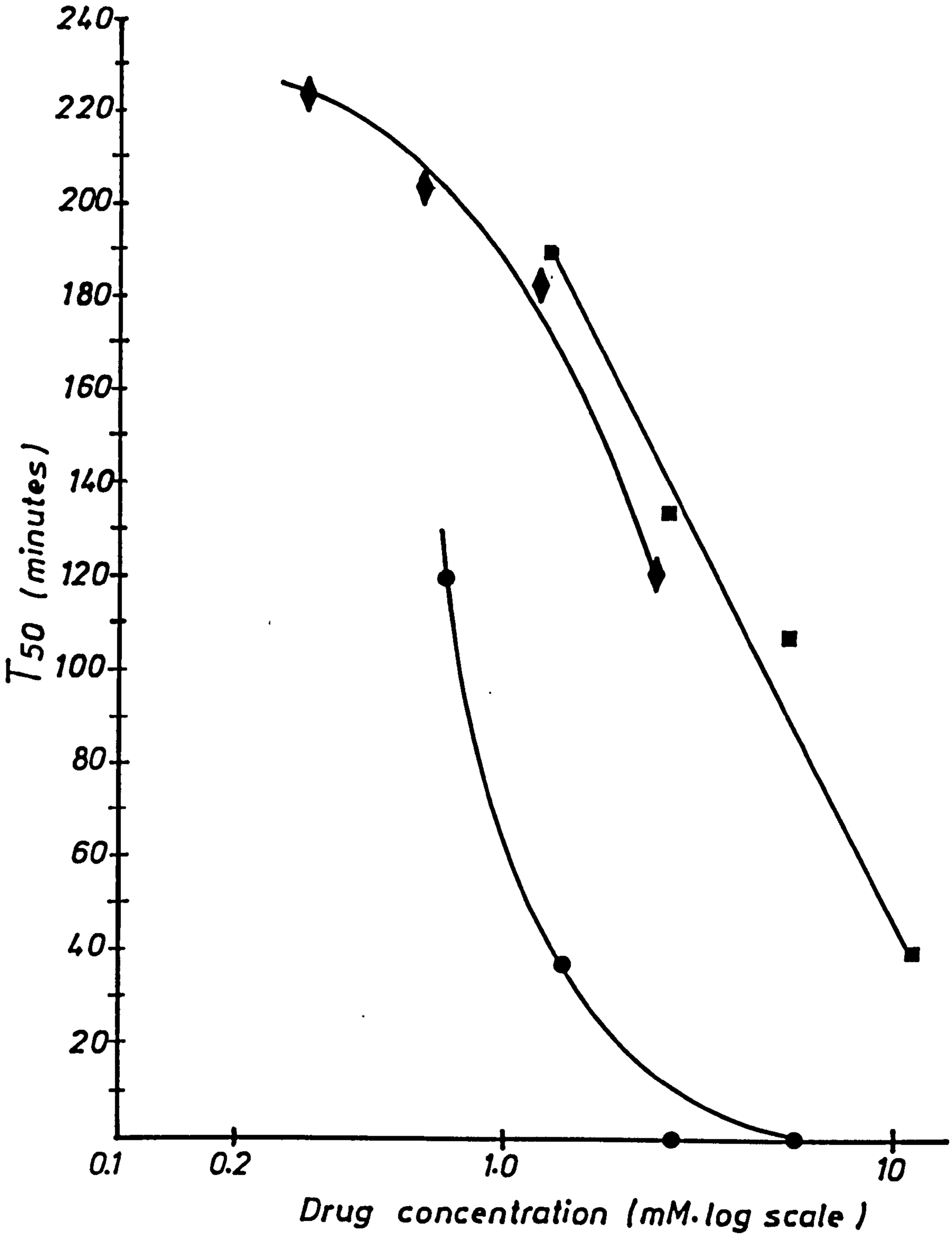

240
220
200
180
160
140
120
100
80
60
40
20
T_{50} (minutes)
0.1
0.2
1.0
10
Drug concentration (mM. log scale)

Figure 6.15 The relationship of estimated times to 50% inhibition of elicited swimming activity (T_{50}) against drug concentration (mM) for the indolealkylamine, 5-hydroxytryptamine (serotonin).

(The T_{50} estimates were obtained graphically from Figure 6.4 with the assumptions concerning activity levels in control cuvettes described in detail in the legend to Figure 6.1.)

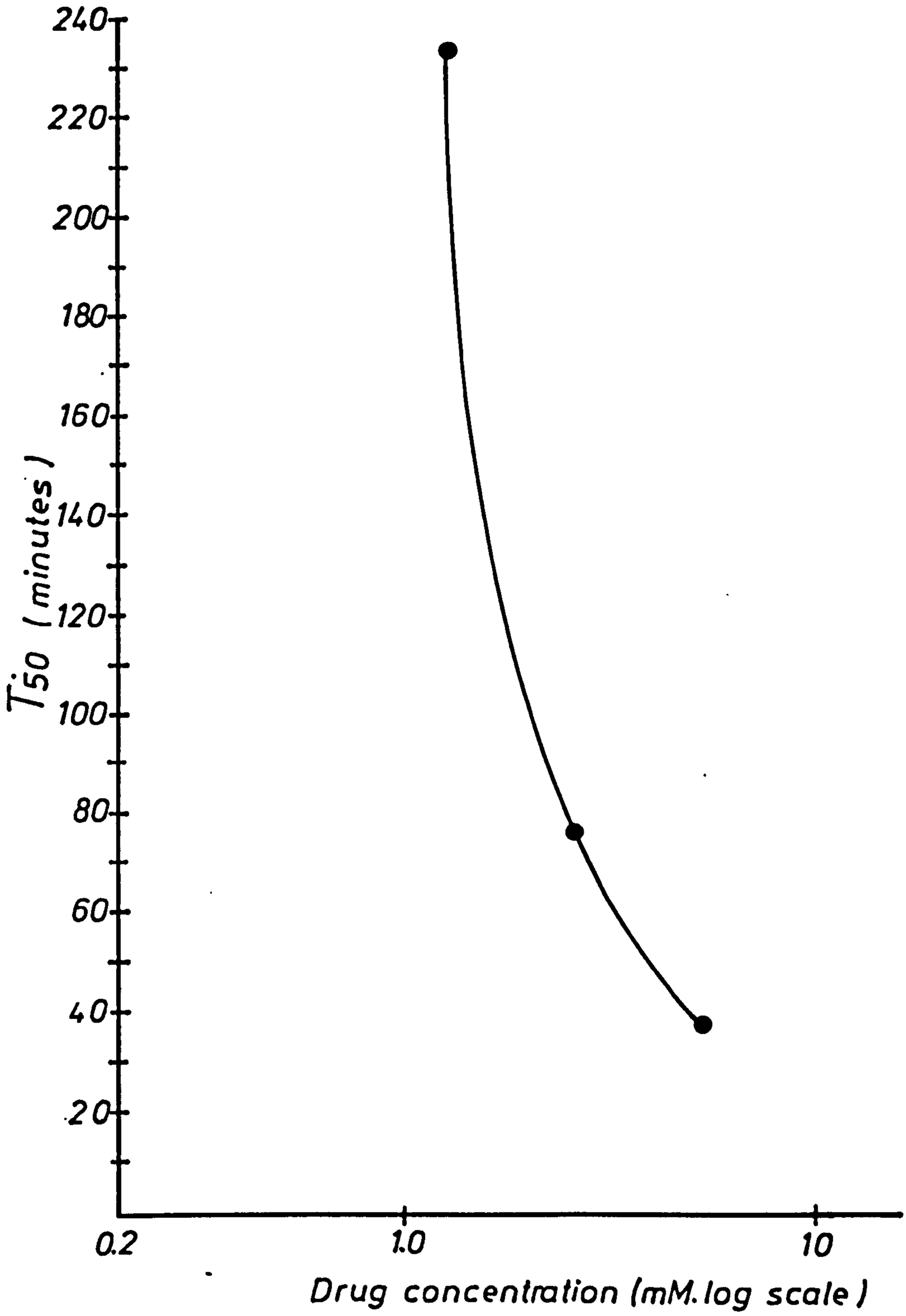

240
220
200
180
160
140
120
100
80
60
40
20
T_{50} (minutes)
0.2
1.0
10
Drug concentration (mM. log scale)

Figure 6.16 The relationship of estimated times to 50% inhibition of elicited swimming activity (T_{50}) against drug concentration (mM) for the post-synaptic cholinergic receptor blockers, atropine, d-tubocurarine, pilocarpine and piperazine.

○ represents atropine

□ represents d-tubocurarine

● represents pilocarpine

■ represents piperazine

(The T_{50} estimates were obtained graphically from Figures 6.9, 6.7, 6.10 and 6.8 with the assumptions concerning activity levels in control cuvettes described in detail in the legend to Figure 6.1.)

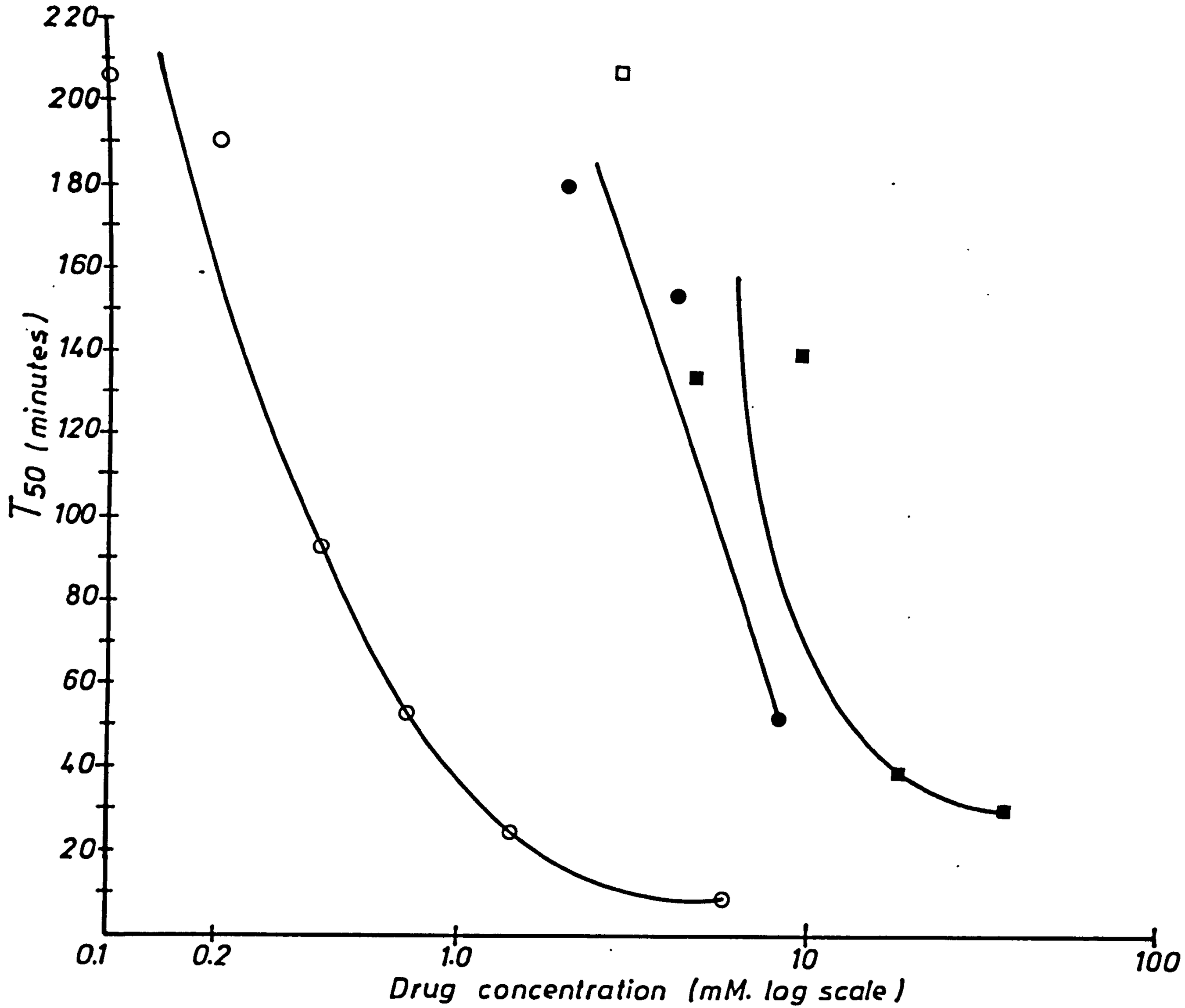

220
200
180
160
140
120
100
80
60
40
20
T_{50} (minutes)
0.1
0.2
1.0
10
100
Drug concentration (mM. log scale)

Figure 6.17 The relationship of estimated times to 50% inhibition of elicited swimming activity (T_{50}) against drug concentration (mM) for the acetylcholinesterase inhibitor prostigmine (neostigmine) as well as acetylcholine itself.

■ represents prostigmine (neostigmine)

● represents acetylcholine

(The T_{50} estimates were obtained graphically from Figures 6.5 and 6.13 with the assumptions concerning activity levels in control cuvettes described in detail in the legend to Figure 6.1.)

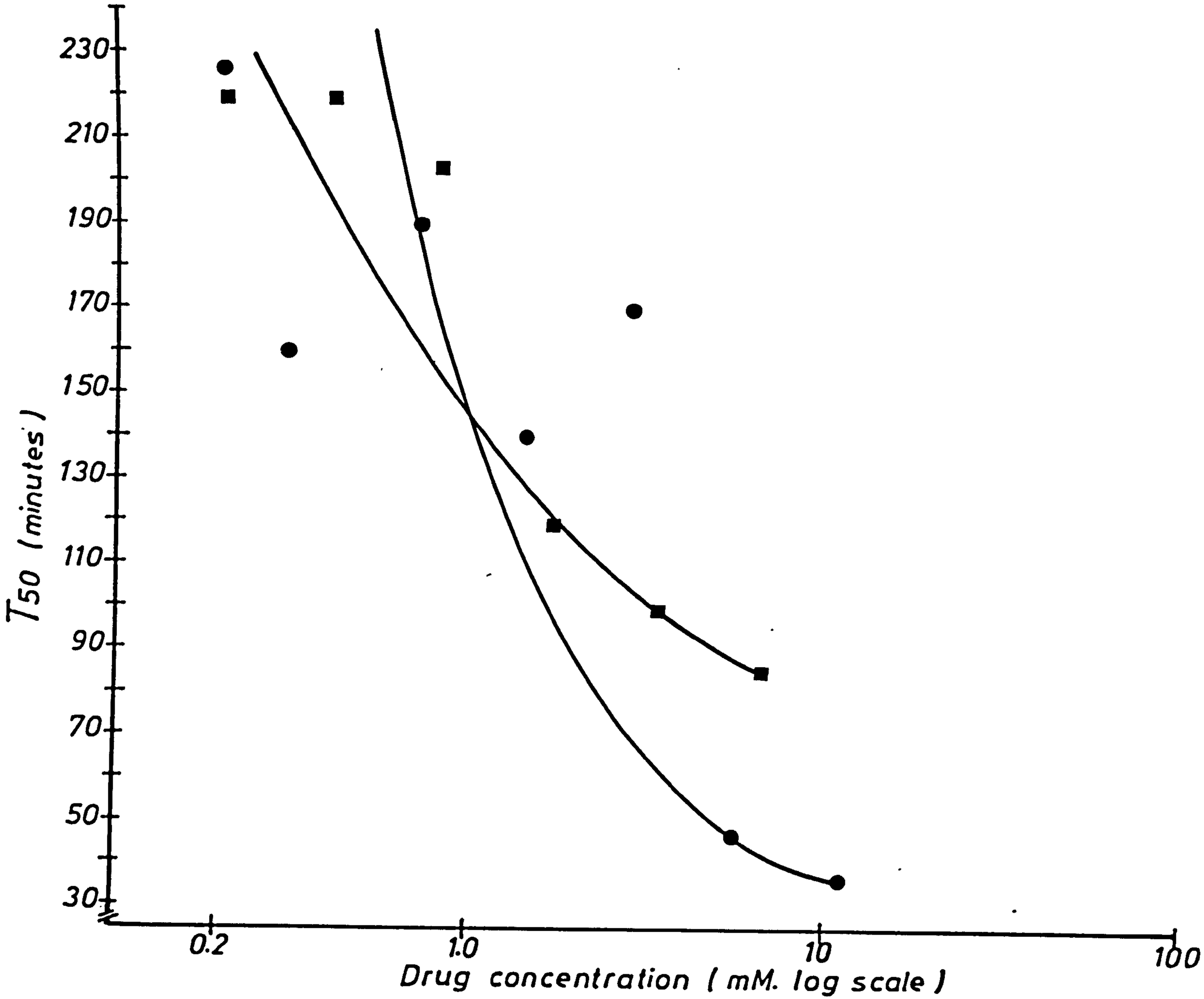

230
210
190
170
150
130
110
90
70
50
30
T50 (minutes)
0.2
1.0
10
100
Drug concentration (mM. log scale)

Figure 6.18 The proportion of separated heads and tails of cercariae (Ps) showing elicited swimming behaviour in physostigmine (eserine) (0.19 mM) and adrenaline (1.37 mM) at varying times after the initiation of drug exposure.

(a) Heads (b) Tails

● represents tapwater control

▲ represents physostigmine (eserine)

□ represents adrenaline

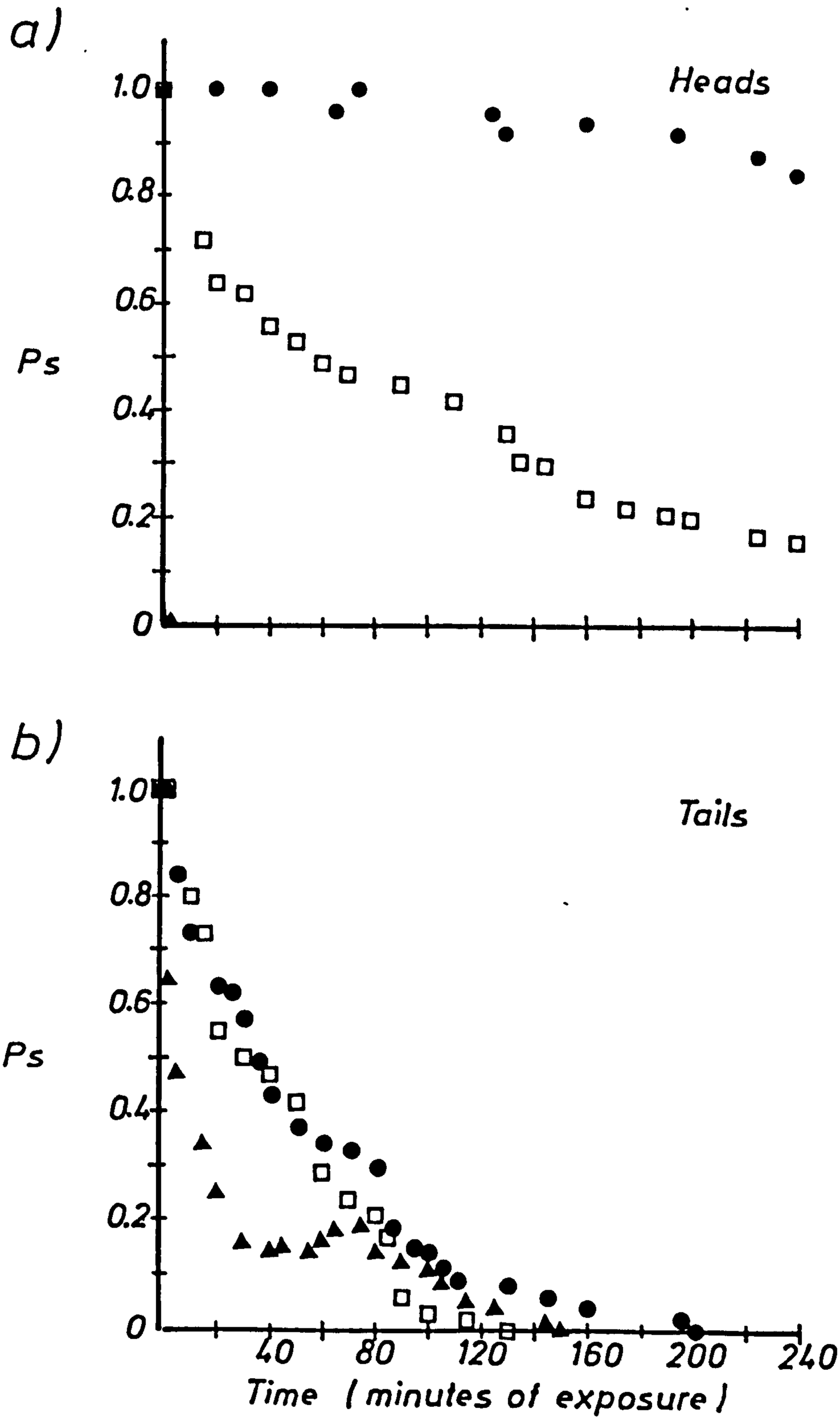

a)
1.0
0.8
0.6
0.4
0.2
0
Ps
Heads
b)
1.0
0.8
0.6
0.4
0.2
0
Ps
Tails
40
80
120
160
200
240
Time (minutes of exposure)

Figure 6.19 The proportion of separated heads and tails of cercariae (Ps) showing elicited swimming behaviour in atropine (5.76 mM) and nicotine (1.23 mM) at varying times after the initiation of drug exposure.

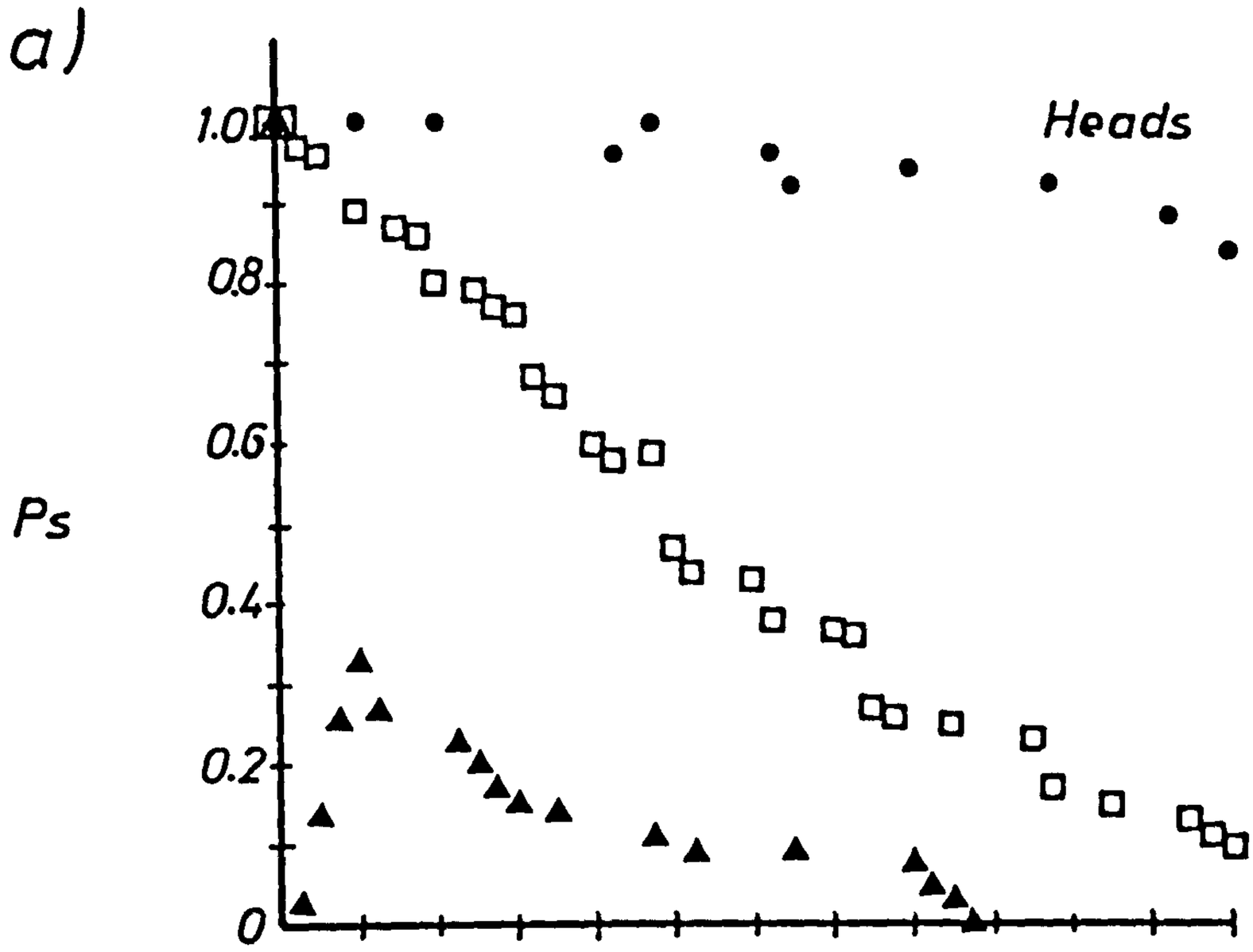
(a) Heads (b) Tails

● represents tapwater control

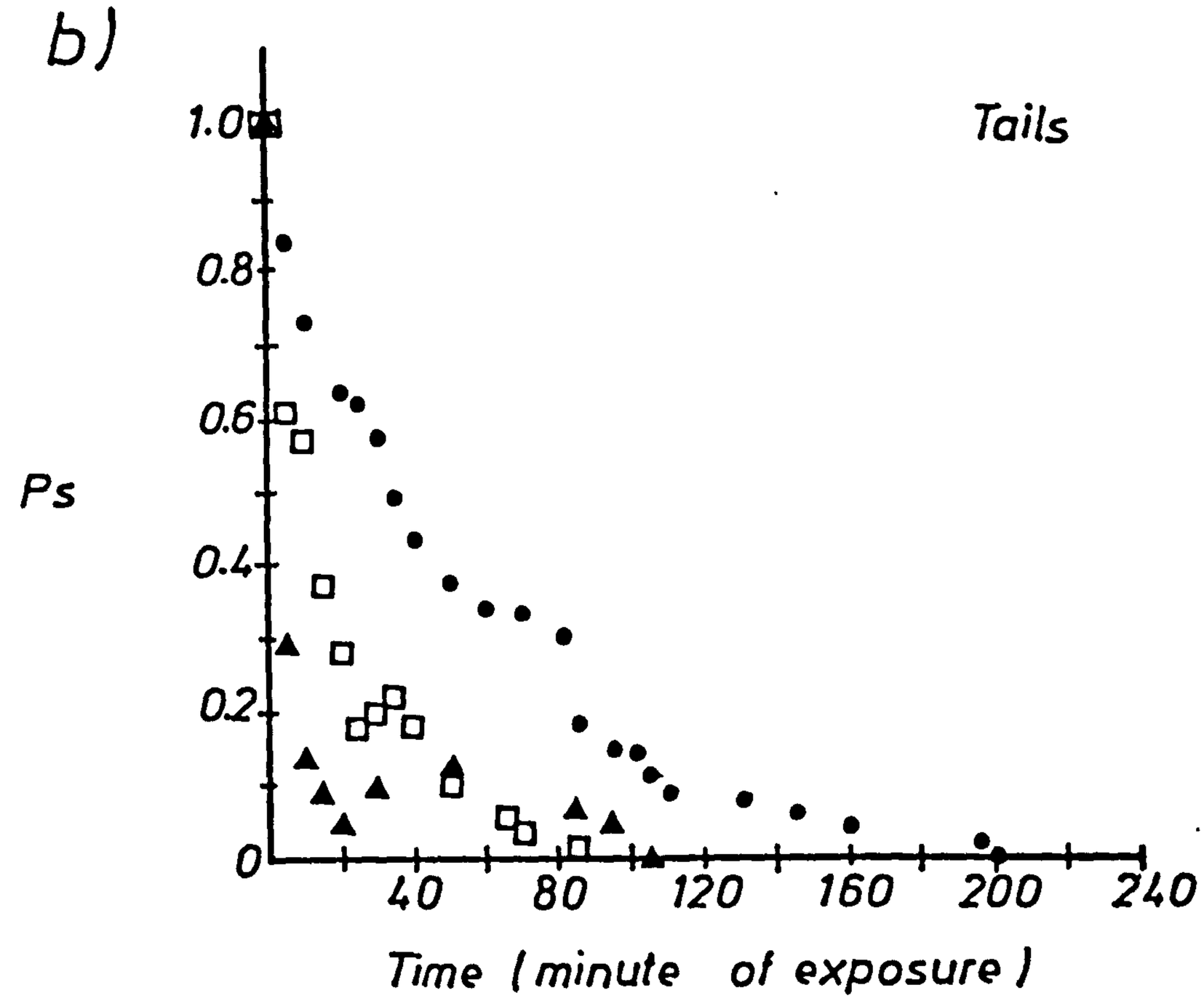
□ represents atropine

▲ represents nicotine

a)


Heads
1.0
0.8
0.6
0.4
0.2
0
Ps

b)


Tails
1.0
0.8
0.6
0.4
0.2
0
Ps
40
80
120
160
200
240
Time (minute of exposure)

with time. Inspection of Figure 6.19 and a comparison of experimental and controls data suggests that 5.76mM atropine has a somewhat more pronounced effect on tails than on heads as the respective times to 50% inhibition for these regions are approximately 20 minutes and 100 minutes.

1.23mM nicotine has effects on both separated heads and tails. In both cases 50% inhibition is reached rapidly (i.e. in less than 10 minutes) but thereafter there is evidence of some reversal of the inhibitory effect.

6.3.3 Drug antagonists

Attempts were made to antagonize the inhibitory effects of physostigmine (eserine), nicotine and piperazine by using drugs which are known to have antagonistic effects upon them (see Chapter 6.2.4). In no case, with either of the antagonist application methods utilized, was any consistent recovery in elicited swimming activity observed.

6.4 Discussion

The experimental design utilized in the investigations described in this chapter could only monitor reductions in cercarial swimming activity. As a consequence of this, interpretation of the significance of the results depends on assumptions about the roles that the applied pharmacological agents might be playing. In this context, it has usually been assumed here that the types of action shown by the drugs in other invertebrates (or vertebrates) is likely to be mimicked in the cercarial situation if any response occurs.

Another general interpretational problem concerns the mode of drug application. Like other workers dealing with small larval helminths (see, for instance, Chance & Mansour, 1953; Barker, Bueding & Timms, 1966; Croll & Alhadithi, 1972) it has been necessary in these experiments to directly immerse intact helminths in the pharmacological agents under investigation. This poses a number of questions. First, if a drug has an effect on larval behaviour, is it mediated via an internal direct interaction with, for instance, synapses or neuromuscular junctions; or is it the result of the sensory equipment of the larvae responding to the externally applied drug? Second, if

a drug appears to have no effect or a minimal influence on larval behaviour, is this lack of response due to an insensitivity of the larvae to this drug or the failure of the drug to penetrate the organism in sufficient concentrations to influence appropriate internal target molecules?

These multiple problems of interpretation are incapable of complete resolution. The pattern of experiments described here, however, does enable some of them to be minimized. Several aspects of the results and the experimental design help in this respect. Firstly, in several cases it has been possible to use a range of drugs in a particular activity group thus enabling the possible penetration problems associated with particular drugs to be assessed in a more general context. Secondly, in cases where rapid and complete cessation of muscular activity occurs, it seems unlikely that this could be due to a sensory input related to the drug. Thirdly, the use of separated heads and tails in these experiments has enabled a more precise estimation of the spatial distribution of a drug response to be produced. With a caution necessitated by the problems outlined above, the results of T. patialense cercarial drug exposure experiments can be discussed below in the context of data from pharmacological experiments on other organisms.

6.4.1 Catecholamines

The catecholamines and, in particular, dopamine would appear to function as transmitters in most if not all invertebrate phyla. Evidence for this conclusion with respect to dopamine is strong for the molluscs and the insects, while noradrenaline appears to play at most a very minor transmitter role relative to its importance in vertebrates. The previous evidence for adrenaline acting as a transmitter in invertebrates is even less substantial than that for noradrenaline (Leake & Walker, 1980). Those findings on invertebrates that do exist for these catecholamines suggest a pattern of effects in which inhibitory actions are most common.

Wells (1937), for instance, reported that adrenaline in concentrations between $1/10^6$ and $1/10^5$ has an inhibitory effect on the activity of the proboscis of the annelid Arenicola marina

if no oesophageal tissue is present. If oesophageal tissue is present, adrenaline has an exciting action. He concluded that the continuous regular series of rhythmical contractions in the musculature of the proboscis is due to impulses flowing forwards from the excited oesophagus. Adrenaline also inhibits the rhythm of circular muscle strips in Arenicola body wall.

Noradrenaline at concentration 10^{-4}M mimics the inhibitary effect of dopamine on the heart beat of the limpet Patella vulgata (Leake, Evans & Walker, 1971). Dopamine at concentration 10^{-4}M was found to inhibit the stimulatory effect of 10^{-4}M 5HT on both the intact and the nerve-muscle preparations of Fasciola hepatica (Mansour, 1957). Dopamine (10^{-9}g/ml) was also found to inhibit the spontaneous contractions of the mollusc intestine, the cardiac portion of the gut, but not the rectum. Higher concentrations (10^{-6}g/ml) induced contractions in all regions of the gut. High doses of noradrenaline and adrenaline mimicked both actions of dopamine (Dougan & McLean, 1970). In addition, adrenaline, noradrenaline and dopamine have been reported to excite the ventral nerve cord of the cockroach Periplaneta americana (Milburn & Roeder, 1962; Hodgson & Wright, 1963).

Some studies have failed to find catecholamines in invertebrates. Adrenaline at concentrations below $2x10^{-3}$ (Chance & Mansour,1953) and adrenaline and noradrenaline at a concentration of $5x10^{-3}$ (Mansour, 1957) failed to produce any effect on intact adult Fasciola hepatica preparations. Baldwin & Moyle (1949) could not detect any effect of adrenaline 10^{-4}(w/v) on Ascaris muscle preparations.

It was known for many years that adrenaline administration into the vertebrate body results in stimulatory as well as inhibitory effects in various tissues. Those conflicting effects were explained by Ahlquist's (1948) hypothesis. He proposed two different adrenergic receptor types (α and β) the stimulation of which results in excitatory and inhibitory effects respectively. β receptor.. are subcategorized into β1 and β2 receptors. The stimulation of β2 receptors results in the relaxation of the smooth muscles of some of the blood vessels and of the bronchial muscles in vertebrates (Goodman & Gilman, 1980).

In the present study all three catecholamines that were utilized (adrenaline, noradrenaline and dopamine) produced marked inhibition of elicited cercarial swimming when utilized at external concentrations above about 10^{-2}M.

Adrenaline was more inhibitory than the other two catecholamines, still exerting a relatively rapid onset inhibition below 10^{-3}M. This finding is in interesting contrast to most of the other invertebrate catecholamines studies described above where dopamine or noradrenaline are usually the most potent catecholamine inhibitory transmitters. Previous studies on digeneans have failed to show any effects of catecholamines on adult worms (Chance & Mansour, 1953; Mansour, 1957).

The inhibitory effect of adrenaline on entire T. patialense cercariae is marked by a profound inhibition of the muscular activity of separated cercarial heads in 1.365×10^{-3}M adrenaline. Interestingly, separated tails appear to be completely unaffected when immersed in the same concentration of this drug. This dramatic difference in responsiveness could apparently be due to either intrinsic susceptibility differences or a differential drug penetration effect. The latter seems somewhat unlikely as the TEM ultrastructural appearances of the distal cytoplasm of the tegument in head and tail are generally similar apart from the presence of spines on the head. One is left with the possibility that adrenaline is acting as an inhibitory transmitter in the T. patialense cercarial head whereas no adrenergic synapses, of an inhibitory character, appear to be present in the tail. It might be that the unstriated muscles of the head bear receptors similar to the β2 inhibitory receptor of vertebrates, whereas the striated tail muscles do not.

It is intriguing to note, however, that striated tail muscle activity is inhibited by adrenaline in the intact cercariae. One assumes that this form of inhibition must be indirect and be the result of inhibitory signals from the central nervous system of the head induced by adrenergic activity there.

6.4.2 Indolealkylamines

5-hydroxytryptamine has been identified in most of the major invertebrate phyla, and it is now evident that the invertebrate tissues especially, nervous tissues are capable of taking either

tryptophan or 5-hydroxytryptophan and converting it to 5-hydroxy-tryptamine (Cardot, 1964). Leake & Walker (1980) were of the opinion that 5HT is clearly involved either directly or indirectly in regulating the activity of the myogenic hearts of Lamelli-branchs and gastropods. They also pointed out that it also appears to be a peripheral inhibitory or relaxing transmitter with respect to adductor muscles in molluscs, and there is also evidence that it may modulate the action of peripheral muscles. They stated also that 5HT is certainly a transmitter in the central nervous system of gastropods, mediating both excitatory and inhibitory effects.

The effects of 5HT on digeneans other than T. patialense and other helminths have been reported on a number of occasions. Mansour (1957), for instance, found that 5HT at concentrations $5x10^{-5}$M and $2.5x10^{-5}$M stimulated the motor activity of adults of the digenean Fasciola hepatica while intact and in nerve-muscle preparations respectively. This stimulatory effect was found to be peripheral and not mediated through the central ganglia. Barker, Bueding & Timms (1966) reported that 5HT as low as $2x10^{-5}$M concentration has a stimulatory effect on the muscular activity of Schistosoma mansoni.

A similar stimulatory effect of 5HT for larval hookworm nematodes has been also reported by Croll & Alhadithi (1972) who found that 5HT at about $25.8x10^{-3}$M greatly increased the activity of Ancylostoma tubaeforme larvae over that of the controls. At about $0.258x10^{-3}$M there was also prolonged stimulation lasting at least 24 hours, but at $0.258x10^{-5}$M larval activity was not significantly increased.

In contrast to the digenean findings of Mansour (1957), and Barker et al (1966), and those on nematodes (Croll & Alhadithi, 1972) the present study suggests that T. patialense cercariae show a moderate degree of swimming inhibition when immersed in concentrations of 5HT above $1x10^{-3}$M. At lower molarities, similar to those used by Barker et.al.(1966) it was difficult to demonstrate significant inhibition. These findings could be taken to suggest that 5HT is an inhibitory transmitter in the T. patialense cercarial system. This is probably a premature conclusion, however, as the Schistosome work of

Barker et al (1966) was also carried out using entire digeneans. It is quite possible that at 5HT concentrations around 10^{-5}M, this drug could be having a stimulatory effect on the cercariae. The experimental design, however, would not be able to demonstrate such an activity. The fact that some inhibition occurs at 5HT concentrations about one hundred times greater than this,might simply be due to pathologically high drug concentrations.

6.4.3 Acetylcholine

Bueding (1952) demonstrated that extracts of Schistosoma mansoni, Litomosoides carinii and Ascaris lumbricoides muscle, contain high acetylcholinesterase and choline acetylase activities. Chance & Mansour (1953) also reported the existence of these compounds in adults of Fasciola hepatica. They also observed that acetylcholine at a concentration of 10^{-3} relaxed and depressed the rhythmical movement of the parasite after a brief contraction, but recovery was spontaneous in the presence of the drug. Acetylcholine at a concentration of $1x10^{-2}$ and $3x10^{-3}$M produced paralysis of the body musculature and of the two suckers of Schistosoma mansoni (Barker et al , 1966). In contrast, acetylcholine at 10^{-6}(w/v) concentration produced a marked stimulatory effect on Ascaris muscle (Baldwin & Moyle, 1949), while Croll & Alhadithi (1972) reported that acetylcholine in a concentration of 10^{-1} and 10^{-3}M has an immediate but relatively unsustained stimulating effect on the activity of Ancylostoma tubaeforme larvae.

In the present study acetylcholine showed an ability to inhibit elicited cercarial swimming activity at concentration above $3.44x10^{-4}$M. This result is most closely comparable with the body wall and sucker muscle inhibition of Schistosoma mansoni adults (Barker et al., 1966) in similar concentrations of the drug. It is difficult to know whether this effect in T. patialense cercariae is the result of effects at nerve-nerve synapses and neuromuscular junctions or a consequence of receptor cell stimulation.

6.4.4. γ-Aminobutyric acid (GABA)

GABA has been shown to have inhibitory effects in crustacean (Kravitz, 1962) and insect (Ray, 1964) nervous systems. In

studies on parasitic nematodes two rather different findings relating to GABA function have been published. Del Castillo (1969) reported that high concentrations of GABA had no effect on intact worms of Ascaris in vitro, although they demonstrated marked inhibitory effects on the myogenic longitudinal body wall muscles in neuromuscular preparation. Croll & Alhadithi (1972), in contrast showed that GABA at concentrations between $97x10^{-3}$M and $9{\cdot}7x10^{-5}$M had no effect on larval hookworm (Ancylostoma tubaeforme) motility or posture when compared with untreated controls.

The results of this study match those of the latter study in that entire cercariae failed to show any consistent inhibition of swimming activity in GABA concentrations up to $7.76x10^{-2}$M.

6.4.5 Post-synaptic cholinergic receptor blockers

All five of the drugs in this category utilized in this study are known to exert considerable blocking activity at post-synaptic cholinergic receptors in animals other than T. patialense.

Atropine at concentrations from 1:5000 to 1:100000 (w/v) has a slightly excitatory action on the annelid body wall (Wu, 1939). In contrast, atropine is without effect at concentration 1:1000 (w/v) on Ascaris (Baldwin & Moyle, 1949) and at the same concentration on intact and degangliated preparations of Fasciola hepatica adults (Chance & Mansour, 1953). Barker et al (1966) found that $2x10^{-4}$M atropine produced hyperactivity of the body wall musculature, oral sucker and ventral sucker in the intact worm as well as in the separated anterior end and the posterior body preparation of adult Schistosoma mansoni. The stimulatory effect was found to persist for several hours and eventually was followed by inhibition. Similarly, Wilson & Schiller (1969) observed that atropine at concentrations of $2x10^{-4}$ and $5x10^{-5}$M, stimulated activity of the scolex and immature regions of six-day-old tapeworms (Hymenolepis diminuta) for the first few minutes of exposure, followed by a condition of flaccid paralysis. Atropine at the same concentrations caused only

slight stimulation of motility in 12 and 15-day-old worms.

In the present study atropine concentrations down to about 10^{-4}M were able to inhibit elicited cercarial swimming activity. The experimental design was not able to reveal any transient activation that might have preceded this inhibition, but, if present, it must have only been of a short duration because in $5.76x10^{-3}$M atropine all cercariae were immobile in less than 20 minutes. The results on separated heads and tails showed that both were inhibited by $5.76x10^{-3}$M atropine but tails more rapidly than heads. Atropine is known to block the same receptor as one used by acetylcholine. Given this knowledge about its mode of action, it is difficult not to conclude that it is inhibiting head, tail and whole cercarial muscular activities by blocking post-junctional acetylcholine receptors on cercarial muscles or similar post-synaptic receptors in the central nervous system. The enhanced inhibition of tails when compared with heads could be the consequence of region specific densities of such receptors, different receptors types or different drug permeabilities.

6.4.6 Nicotine

The effects of externally applied nicotine have been noted on both digenean and nematodes in previous publications. Chance & Mansour (1949), for instance, found that nicotine at a concentration of $2:10^{-5}$(w/v) had a paralytic effect on adult Fasciola hepatica, while nicotine at $1:10^{5}$(w/v) and $1:10^{7}$(w/v) had no such effect on adult Ascaris lumbricoides (Baldwin & Moyle, 1949). Barker et al (1966) reported that nicotine at concentrations 10^{-7}M to 10^{-3}M lacked an effect on the motor activity of Schistosoma mansoni adults. Nicotine at concentrations of $6x10^{-4}$ to $6x10^{-6}$M had a stimulatory effect on Ancylostoma tubaeforme larvae (Croll & Alhadithi, 1972). He reported that the effect did not persist, however, at $6x10^{-4}$M nicotine appeared to damage the larvae.

In the present study, concentrations between $1.23x10^{-4}$ and $2.47x10^{-3}$M were used. At the upper end of this range nicotine had a rapid and irreversible paralytic effect on cercariae while at lower concentrations some reversal of this effect with time was apparent. In this respect, T. patialense cercariae

are responding to nicotine in the times that adult Fasciola hepatica do (Chance & Mansour, 1949), but differ from the insusceptibility of Schistosoma mansoni (Barker et al ,1966).

It is known from vertebrate studies that nicotine binds to cholinergic receptors. It is also recognized that at low concentrations it can induce depolarization and excitation of the receptor bearing membrane while at higher concentrations this effect leads to a depolarization block (Lewis, 1965). The present findings on T. patialense cercariae can probably be usefully examined in the light of these aspects of nicotine pharmacology. It is likely, for instance, that the irreversible inhibition in 2.47×10^{-3}M nicotine is the result of a depolarization block of cholenergic receptor. The partial reversal of an initial inhibition at lower molarities can probably be explained by a time-dependent pattern of nicotine induced receptor activation.

Experiments reported in this chapter have shown that 1.23×10^{-3}M nicotine inhibits both separated heads and tails of T. patialense cercariae. In both cases the inhibition is partially reversed through time. These results suggest that cholinergic receptors are present in both parts of the larvae.

6.4.7 d-Tubocurarine

Although Croll & Alhadithi (1972) have demonstrated that 14.37×10^{-2}M d-tubocurarine activated Ancylostoma tubaeforme larvae, experiments on neuromuscular preparations of Ascaris (Baldwin & Moyle, 1949) showed that the same drug could produce complete paralysis. In studies on digeneans, it has been reported that 10^{-3}(w/v) concentration d-tubocurarine did not affect the motor activity of adult Fasciola hepatica (Chance & Mansour, 1953), nor did it influence the activity of Schistosoma mansoni adults in a concentration range of 5×10^{-5} to 1×10^{-2}M (Barker et al ,1966). Lewert & Hopkins (1965) were equally unable to demonstrate any effect of the drug on the activity of S. mansoni cercariae.

In the present study a slight inhibitory effect of 2.88×10^{-3}M d-tubocurarine was apparent on the elicited swimming activity of T. patialense cercariae. Given the effectiveness of atropine and nicotine in inducing inhibition of this activity, the relative ineffectiveness of d-tubocurarine in this context in

both S. mansoni (Lewert & Hopkins, 1965) and T. patialense cercariae is probably due to a permeability barrier.

6.4.8 Pilocarpine

Externally applied pilocarpine appears to have no effect on the motor activity of adult worms of Ascaris (Baldwin & Moyle, 1949), Fasciola hepatica (Chance & Mansour, 1949) and Schistosoma mansoni (Barker et al , 1966).

The finding of a moderate and consistent dose-dependent inhibitory effect on T. patialense cercariae in this study is therefore of interest, with significant inhibition (i.e. more than 50% inhibition within four hours) occurs down to about $2x10^{-3}$M pilocarpine. This result is completely compatible with those for atropine and nicotine as,in vertebrates, pilocarpine is known to act as an inhibitor of cholinergic activity by blocking receptors or end organs innervated by post-ganglionic cholinergic nerves (Lewis, 1965).

6.4.9 Piperazine

The great bulk of the helminth-directed pharmacological work with piperazine has been concerned with nematodes because a range of piperazine compounds have proved to be cheap and efficient chemotherapeutic agents for use against intestinal nematodes.

Goodwin & Standen (1954) noticed that parasitic nematodes expelled following treatment with piperazine were narcotized motionless and relaxed, but still alive. These changes can be reversed if the worm is immersed in Ringer's solution at 37°C. Utilizing the free-living nematode Caenorhabditis briggsae, Fiakpui (1967) observed a toxic effect of piperazine over a wide range of concentrations (20 - 0.04mM). The extent of paralysis was generally concentration-dependent with paralysis being more pronounced at higher concentrations. He suggested that piperazine acts as a competitive blocking agent of receptor sites at the neuromuscular junctions similar to that produced by d-tubocurarine. These observations are compatible with those of Norton & DeBeer (1957) who showed that piperazine caused paralysis of Ascaris lumbricoides neuromuscular preparations and blocked the response of the muscle to acetylcholine. Electrical stimulation of the same muscle could cause contraction and was thus not blocked by piperazine. Norton & DeBeer concluded that the action of piperazine was on the neuromuscular junction rather than directly

on the muscle.

Given this background of data on the mode of action of piperazine, the present finding that the drug shows an inhibitory effect on T. patialense swimming activity is not altogether suprising.

When one considers the results presented in this chapter on five different cholinergic receptor blockers, a remarkably consistent pattern appears to have emerged. There are differences in the molar thresholds of drug activity for the different agents and nicotine does demonstrate a partial reversal of inhibition through time. All the drugs, though, show a repeatable ability to inhibit the elicited swimming of T. patialense cercariae. This central fact points to the conclusion that cholinergic receptors must be present in these larvae and must be very important in their neural and neuromuscular organisation. It is also very likely that in general the cholinergic nerves must be excitory in their influence on motor activity.

6.4.11 Acetylcholinesterase inhibitors

From the experimental results relating to cholinergic receptor blockers (see above) it seems clear that acetylcholinesterase inhibitors might be expected to have a considerable influence on cercarial activity if they were able to penetrate to the synapses and neuromuscular junctions where acetylcholinesterase might be assumed to be operating.

Previous experiments in which acetylcholinesterase inhibitors were applied to parasitic platyhelminths have usually showed the drugs to have had potent effects on motility. Chance & Mansour (1953), for example, reported that the whole Fasciola hepatica adults as well as the deganglionated preparations were very sensitive to the action of physostigmine at concentration above $1:10^{3}$(w/v). The response was characterised by either complete cessation of movement or by marked depression of the amplitude and rate of rhythmical movement. Similarly, Lewert & Hopkins (1965) noted that physostigmine at concentration as low as $1.2x10^{-3}$M paralysed Schistosoma mansoni cercariae for up to 45 minutes, during which time infrequent erratic twitchings of the anterior end occurred. After a period of paralysis, the cercariae resumed activity almost equal to that of the controls. Physostigmine at concentrations 10^{-5} M and $2x10^{-6}$ M and prostigmine at

10^{-3}M and $5x10^{-5}$M have been reported to produce paralysis in adult Schistosoma mansoni body musculature and suckers respectively (Barker et al, 1966). They found that paralysis occurred within two minutes of exposure to these drugs. In Hymenolepis diminuta (Wilson & Schiller, 1969) found that prostigmine at a concentration of $5x10^{-5}$M and physostigmine at a concentration of $2x10^{-5}$M caused an immediate depression in the motility of the scolex of six-day-old worms. They reported that normal activity resumed after 16 minutes in prostigmine while only slight motility resumed after 1.25 hours in physostigmine.

In the present study, both physostigmine and prostigmine were capable of inducing profound inhibition of cercarial swimming. The inhibition of prostigmine was of a relatively simple time- and dose-dependent form, while that caused by physostigmine although very potent in the short term, revealed some reversability. Verymarked inhibition was still being caused by physostigmine at concentrations as low as $2.4x10^{-5}$M. These findings add further weight to the conclusion that cholinergic synapses and neuromuscular junctions must have an important role in T. patialense cercariae.

The studies on separated heads and tails using physostigmine revealed that heads were considerably more susceptible to this drug than tails. The former regions demonstrated an almost instantaneous cessation of all muscular activity in the presence of $1.9x10^{-4}$M physostigmine.

The drug exposure experiments on T. patialense cercariae provide one of a range of strands of evidence concerning the organisation of neural and neuromuscular control on these larvae. The other major strands are histochemical and ultrastructures. Taken together the pharmacological findings of this chapter appear to suggest some general conclusions about transmitter function in this system, namely:

(i) catecholamines are important inhibitory transmitters, and it is likely but not certain that adrenaline is the principal transmitter of this type;

(ii) cholinergic synapses and/or neuromuscular junctions play an important role in the larvae and are probably excitatory in character;

(iii) 5HT perhaps has a minor role as an inhibitory transmitter; (iv) GABA is unlikely to have any transmitter function at this stage of the life cycle.

The only finding which seriously casts doubt on any of these conclusions is the fact that externally applied acetylcholine itself causes moderate inhibition of locomotor activity. This result is perhaps unexpected in view of the weight of evidence from experiments on cholinergic receptor blockers and acetylcholinesterase inhibitors, which suggest that its overall effect should be excitatory. The paradoxical evidence of acetylcholine induced inhibition may be an indirect effect, unrelated to the postulated excitatory transmitter function. The studies on acetylcholinesterase inhibitors in this chapter show pharmacological evidence for the presence of acetylcholinesterase in cercariae and Chapter 5 has provided ample histochemical evidence for the extensive distributions of this enzyme throughout the nervous system. Given these facts, it is very likely that externally applied acetylcholine should have little opportunity to influence motor activity directly because it would be hydrolyzed by acetylcholinesterase before it reached receptor sites. Perhaps it is more likely that the external acetylcholine is influencing surface receptor activity in such a way as to induce the central nervous system to send inhibitory signals to muscle effectors.

CHAPTER 7

Ultrastructural investigations on the musculature of Transversotrema patialense cercariae with particular reference to that of the tail

7.1 Introduction

The two main types of free-swimming larvae in the life cycles of typical digeneans are the miracidia and cercariae. Their principal modes of locomotion are characteristically different. Miracidia normally move using ciliated surface cells. Cercariae on the other hand, after emergence from their molluscan first intermediate host, usually swim in the external environment using muscular undulations of an elongate tail. The active swimming activity, sometimes interspersed with periods of passive dropping or no major movement, carries cercariae to the hosts which they will infect (second intermediate or final hosts) or to substrates on which they will encyst.

Transversotrema patialense cercariae, during the dispersive phase between the snail and the final fish host, exhibit three distinct behavioural modes, namely active tail-first swimming, passive dropping and resting on the substrate (Whitfield, Anderson & Bundy, 1977).

The rate of tail beats was found by Bundy (1981a) to be 30 Hz at 24°C enabling the larvae to develop a speed of 25.9 mm/sec (Whitfield et.al.,1977). One might, therefore, expect that the tail muscles would show structural adaptation related to the capacity to sustain such rapid rates of undulation.

Several previous authors have described the musculature of cercarial tails in a range of species using light microscopical techniques. These studies include observations by Olivier (1940) on Apharyngostrigea pipientis; those of Pearson (1956, 1959, 1961) on Alaria arisaemoides and Alaria canis; Strigea elegans and Neodiplostomum intermedium respectively and that of Dönges (1964) on Posthodiplostomum cuticola.

Using the electron microscope several workers have made more detailed observations on the structure of cercarial caudal musculature. Among such ultrastructural studies are those of Kruidenier & Vatter (1958) on Schistosoma mansoni and Tetrapapillatrema concavocorpa; Cardell & Philpott (1960) on Himasthla quissitensis; Lumsden & Foor (1968) on Heterobilharzia americana; Rees (1971) on Parorchis acanthus; Chapman (1973) on Himasthla secunda and Cryptocotyle lingua; Nuttman (1974) on Schistosoma

mansoni; Rees (1975) on Cryptocotyl lingua; Reger (1976) on Schistosoma sp and Sundararaman & Nadakal (1979) on Cercaria chackai.

The presence of apparently striated muscle fibres in cercarial tails has been commented on by many of the above authors. The arrangement, however, of the longitudinal muscle cells appears to vary between different cercariae and the precise arrangement may possibly be correlated with the different modes of swimming of the various different larvae. Apart from the detailed reconstructions of cercarial tail musculature produced by Pearson (1956; 1961) the precise muscular organisation of tails including the orientation of their longitudinal muscles in different regions of the tail has rarely been described.

The purpose of the present investigation was to study the organisation of the tail muscular system of T. patialense cercariae and to relate it, where possible, to the specific functions of the different regions of the tail. Less comprehensive observations on the muscular organisation of the head are also recorded.

7.2 Materials and Methods

The newly shed cercariae were collected from infected snails as described in Chapter 2 (2.3). The cercariae were fixed immediately after collection in 2.5% glutaraldehyde at 4°C. Individual specimens were then prepared for TEM (Chapter 2.5).

Some cercariae were fixed in a 2% aqueous solution of potassium permanganate for 45 minutes at 4°C. Permanganate fixation had the effect of oxidizing and extracting almost all cellular inclusions. Membrane structures, however, were conserved and such preparations proved useful in the interpretation of complex membranous inclusions within muscle cells. After such fixation the specimens were rinsed in two changes of distilled water, two minutes each, dehydrated and embedded in epoxy resin as described above.

Cercariae were studied alive using phase contrast microscopy, particularly in order to investigate the relative

orientations of muscle systems to one another in life.

7.3 Results

7.3.1 Body wall musculature of the head

The muscular organization of the body wall of the head of T. patialense cercariae conforms to a relatively simple pattern. There is an outer circular layer oriented at right angles to the longitudinal axis of the head lying below the basal lamina and the fibrous interstitial matrix and composed of closely packed fibres. These overlie a basically similar layer of longitudinally oriented fibres, arranged in three or four sets of obliquely distributed fibres. The fibres from the innermost layers of body wall muscles appear to pass dorso-ventrally through the flattened body of the cercarial head (Plate 7.1A). In many instances (see Plate 7.1C) these fibres are closely associated with the central nervous system.

All the above muscle fibres are of unstriated type and contain longitudinally arranged thick and thin myofilaments with no real evidence of lateral register between myofilaments (i.e. no striation) (Plates 7.1A and B). Dense bodies are scattered through the myofibres as is characteristic of slow-contracting muscles. Those dense bodies at the periphery are associated with shallow indentations of the sarcolemma (Plate 7.1C). The thin myofilaments are apparently attached to these bodies as is the case in vertebrate smooth muscle and many invertebrate muscles (Lowy & Hanson, 1962; Morita, 1965; Lumsden & Byram, 1967; Lumsden & Foor, 1968; Jamieson, 1981).

Mitochondria and cisternae of the sarcoplasmic reticulum are distributed in the peripheral regions of these cells, beneath the sarcolemma (Plate 7.1C). Scattered glycogen particles are found in the peripheral cytoplasm of each myofibre. The muscle cell bodies are presumably sunk below the level of the muscular contractile regions and have not been clearly identified in this study.

Specialised arrangements of unstriated muscles are associated with, for instance, the oral sucker, ventral sucker and reproductive system ducts. Observations on these muscles are not recorded here.

7.3.2 Gross organization of the tail musculature of T. patialense cercariae

The musculature of the tails of all cercarial types which actively swim show profound modifications for this function. Preeminent among these specialisations is the presence of striated longitudinal muscles which in most species appear to constitute the main propulsive system (Olivier, 1940; Pearson, 1956; 1961; Lumsden & Foor, 1968; Nuttman, 1974; Rees, 1975). Other, non-striated muscles, however, also become modified in structure and location in ways associated with swimming activity.

The tail of a T. patialense cercaria is a complex one with both terminal furcae and proximal arm processes (see Figure 7.1). The three-dimensional muscle plan of the tail is formed from four rather different muscle sets:

7.3.2.1 circular non-striated muscle cells

7.3.2.2 longitudinal non-striated muscle cells

7.3.2.3 dorso-ventral non-striated muscle cells

7.3.2.4 longitudinal striated muscle cells

All these muscle cell types can be seen for instance in a transverse section of the anterior stem region (Figure 7.7).

The actual arrangements of the muscle cells is complex and regionally differentiated within the tail and these aspects will be examined in detail later. It is usually obvious, however, that the non-striated circular muscles are the outermost muscle layer, closely subtending the tegument. Within this circular layer lie the dorso-ventral non-striated and the two types of longitudinal muscles. Of these, the small blocks of non-striated muscles are positioned dorsally and ventrally and the striated muscle cells take up much of the rest of the circumference in a transverse section.

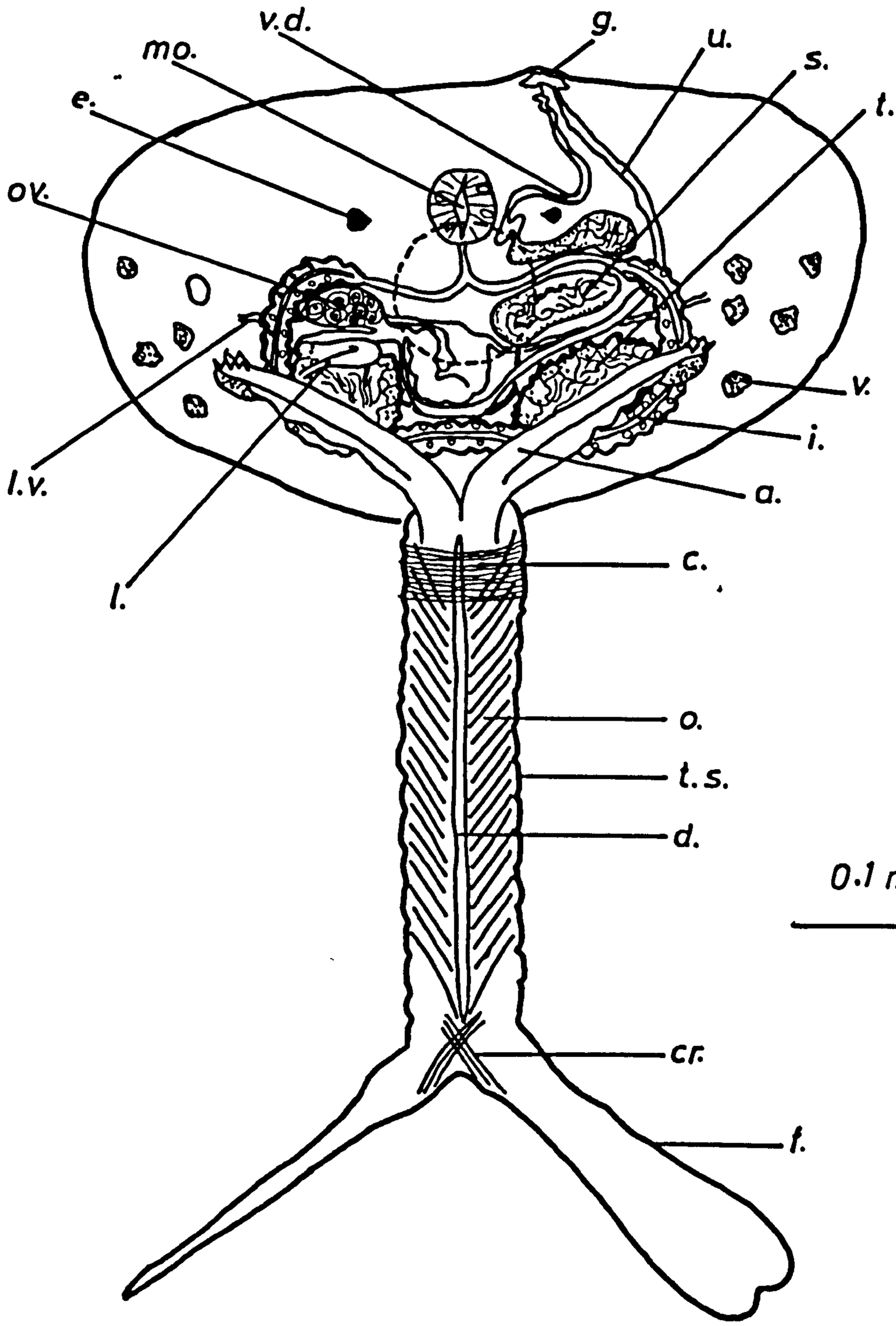
It will be appropriate,at this point, to describe the general ultrastructural organisation of these four muscle cell types before embarking on any more elaborate explanation of how they are organised in the tail itself.

7.3.2.1 Circular non-striated muscle cells

A layer of circular muscle cells lie below the basal lamina and at right angles to the longitudinal axis of the tail (Plate 7.2A). The contractile regions of the circular fibres each

Figure 7.1 Diagrammatic representation of the main morphological features of a fully developed cercaria of T. patialense. Dorsal view with the right tail furca rotated through 90° to reveal its facial appearance. (Based on a diagram in Whitfield, Anderson & Moloney, 1975)

a: arm process; c: circular muscle of the tail stem; cr: cruciform furcal muscles; d: dorsal muscle band of tail stem; e: pigment cup eye; f: tail furca; g: genital opening; ie: intestine; L: Lauren's canal; lv: lateral vitelline duct; mo: mouth; o: oblique longitudinal muscles of cercarial tail; ov: ovary; s: seminal vesicle containing mature motile spermatozoa; t: testis; ts: tail stem; u: uterus; v: developing vitelline cell; vd: vas deferens


v.d.
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c.
l.
o.
t.s.
d.
0.1 mm
cr.
t.

measure approximately 1.5 μm x 0.4 μm in transverse section of the muscle (Figure 7.2; Plate 7.2B).

It has not been possible to decide whether individual circular muscle strands are connected longitudinally or are each connected to their own nucleated cell body. Such bodies have not been unambiguously identified. The circular muscle strands are separated from one another and from the overlying basal lamina by a distinct cell membrane or sarcolemma.

The myofibres contain two types of myofilaments parallel to the longitudinal axis of the fibre (Plate 7.2A), thick myofilaments which appear circular in cross section with a diameter of approximately 25nm, and, irregularly arranged between them, thin myofilaments (Figure 7.2; Plate 7.2C). No striations are apparent and fusiform dense bodies are scattered throughout the myofibres (Figure 7.2; Plate 7.2A). At the periphery of the muscle cells, these dense bodies often become closely linked with shallow intuckings of the sarcolemma and occasionally appear to be attached via these to the basal lamina and the basal membrane of the tegumental distal cytoplasm (Plate 7.2C).

Profiles of sarcoplasmic reticulum are distributed along the periphery of the myofibres close to the sarcolemma (Plate 7.2A and B). No fusion between the sarcoplasmic reticulum and the outer cell membrane has been observed; though the space between the two membrane systems is often very small. Mitochondria about 0.3-0.45 μm in diameter are present near the periphery of these cells, usually on the inner side of the myofibres (Plate 7.2A and C). Glycogen particles are abundant in the sarcoplasm around and among the myofilaments (Plate 7.2A).

7.3.2.2 Longitudinal non-striated muscle cells

These muscle cells form two groups, one mid-dorsally positioned and the other mid-ventral, in sites lying immediately beneath the circular muscle layer. Each group consists of four to eight closely adjacent fibres in any transverse section. (Figure 7.3; Plate 7.3A). Once again, it is difficult to decide conclusively whether or not these fibres connect up at any level along the length of the tail.

The myofibres are morphologically similar to the circular non-striated muscle cells, in respect of their lack of striation

Figure 7.2 Schematic diagram of a peripheral portion of a T. patialense tail stem showing the circular non-striated muscle fibres in transverse section.

cm: circular non-striated muscle fibre; db: dense bodies; m: mitochondrion; n: nucleus of striated muscle fibre; t: tegument; tk: thick myofilament; tn: thin myofilament

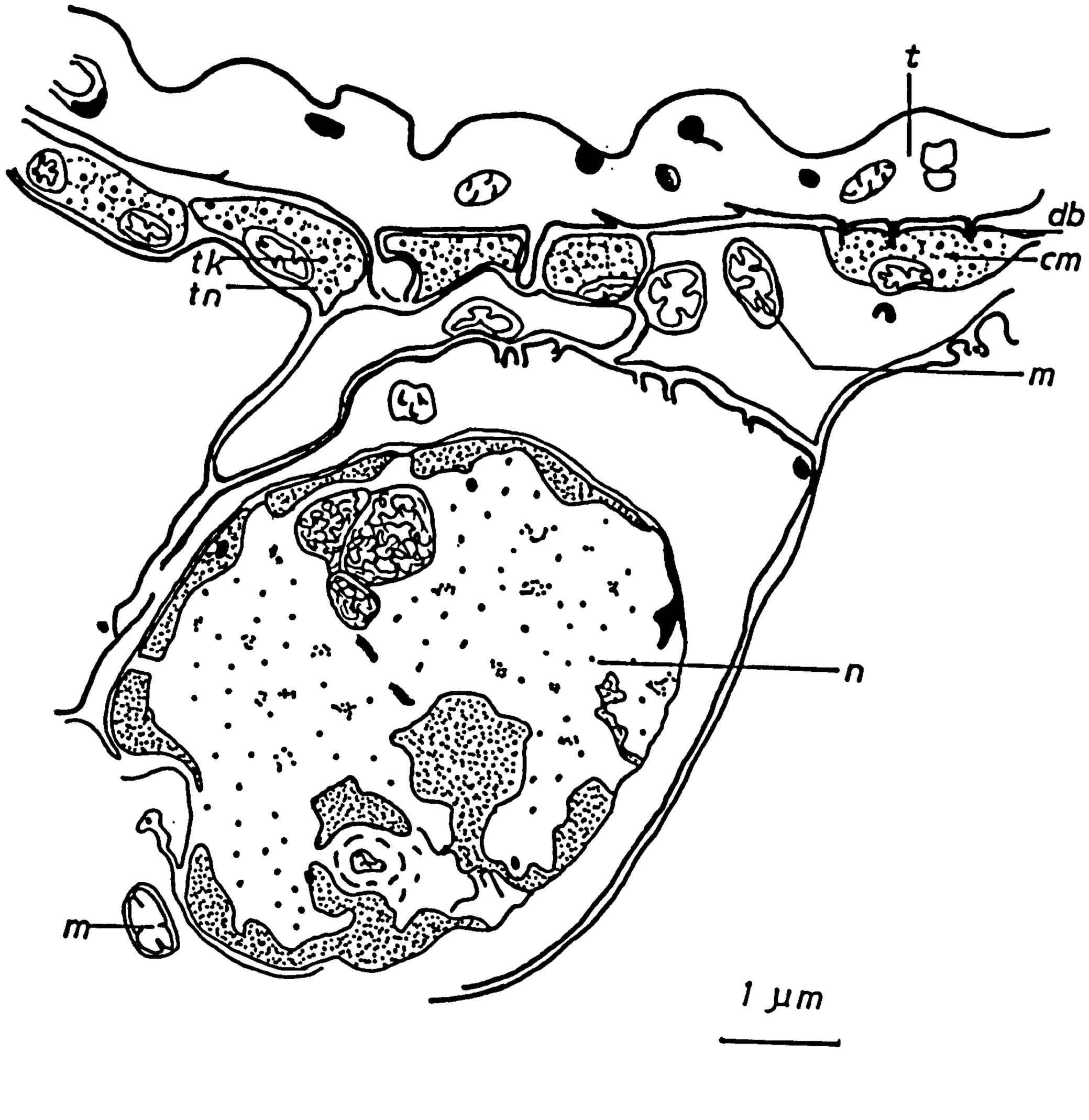
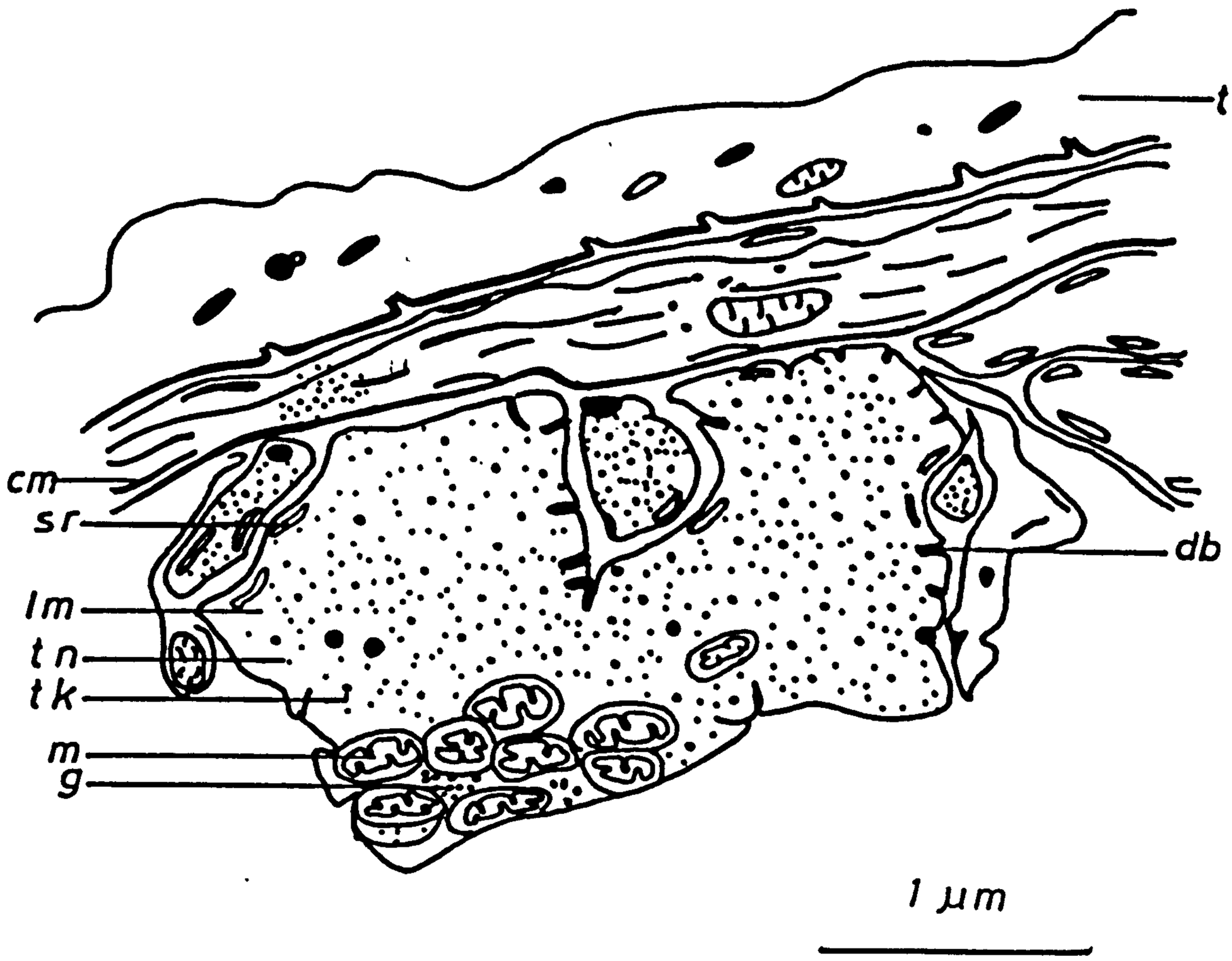

t
db
cm
tk
tn
m
n
m
1 µm

Figure 7.3 Diagrammatic transverse section of a portion of a T. patialense cercarial tail stem showing the circular and longitudinal non-striated muscle cells.

cm: circular non-striated muscle fibres; db: dense bodies; g: glycogen; lm: longitudinal non-striated muscle fibres; m: mitochondrion; t: tegument; tk: thick myofilaments; tn: thin myofilaments; sr: cisternae of sarcoplasmic reticulum


t
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and the organisation of myofilaments, dense bodies, mitochondria and glycogen granules (Figure 7.3; Plate 7.3A). Cisternae of sarcoplasmic reticulum are also encountered immediately beneath the sarcolemma, with slightly dense material between the two membranes (Figure 7.3; Plate 7.3A). The intercellular space bounded by the sarcolemmae of adjacent fibres is usually very narrow (about 15 nm). In places, however, it is wider and here is filled with fibrous material which can take on a striated appearance consisting of alternate dark and light bands (30–35 nm across) arranged at right angles to the longitudinal axis of the muscles (Plate 7.3B). Such enlarged intercellular gaps are often the location of membrane-associated dense bodies which appear to induce hemidesmosomal links between the sarcolemma and the fibrous extra-cellular material. No connections of the longitudinal non-striated muscle to nucleated cell bodies were visible; they must presumably be some distance from the contractile elements and connected to them via narrow cytoplasmic extensions.

7.3.2.3. Dorso-ventral non-striated muscle cells

A sheet of non-striated muscle strands run vertically between the dorsal and ventral blocks of longitudinal non-striated muscle cell in the proximal region of the tail stem. The dorsal and ventral ends of the sheets break into approximately four strands in transverse section of the tail stem and these subdivisions penetrate between the longitudinal non-striated muscle fibres, to be inserted below the tegument.

The general ultrastructural organization of these muscle strands is similar to that of the other two types of non-striated muscle of the tail (Plate 7.8). Cisternae of a sarcoplasmic reticulum have not been observed, however, neither are connections with nucleated cell bodies obvious.

7.3.2.4 Longitudinal striated muscle cells

(a) Cellular organisation

Most of the longitudinally oriented muscle cells of the tail are striated. Such cells are arranged into four discrete groups, two dorso-laterally and two ventro-laterally, extending throughout the greater part of the tail.

Each muscle cell has a contractile region, which is myofilamentous and restricted to a peripheral zone of muscle cell cytoplasm. This region normally lies near the periphery of the tail and is sheathed by a fenestrated sarcoplasmic reticulum. An inner non-contractile sarcoplasmic region or cell body contains the nucleus, mitochondria, glycogen and other organelles. Cytoplasm similar to that of the cell body extends into the central part of the contractile region.

These muscle cells are found to possess a unique structure among the platyhelminths in that they have a striated organisation. The striations are due to the alternation of dark and light bands formed by the spatially ordered presence of two types of myofilaments: thick and thin. The myofilaments are in fact slightly staggered in a regular manner so that a line drawn through corresponding points on adjacent filaments usually forms an angle with the filament's axis somewhat less than the right angle seen in normal cross-striated muscle. This characteristic makes the striated muscles of the cercarial tail similar to the oblique striated muscles found in many other invertebrates (Rosenbluth, 1965a; Heumann & Zebe, 1967; Mill & Knapp, 1970a).

A three-dimensional model is proposed which can account for the appearance of the contractile elements of striated muscles in different planes (Figure 7.4). The three-dimensional complexity of these striated muscles, and their multiplicity of parallel fibrillar elements makes unambiguous textual descriptions of their ultrastructure difficult. Jamieson (1981) has utlized a simple yet useful scheme for overcoming this problem while describing the muscle ultrastructure of oligochaetes. He has proposed that the axis parallel to the myofilaments he termed the Z axis, that parallel to the Z rods and tubules the Y axis and finally that which is mutually at right angles to the other two, the X axis. The latter is therefore at right angles to the myofilaments and tangential to the cell surface of the contractile region. These axes are indicated in Figure 7.4 and will be used when appropriate below to define muscle axes and planes.

The contractile outer zone of such cells has a relatively constant width (i.e. approximately 0.7 μm) which can be visualised

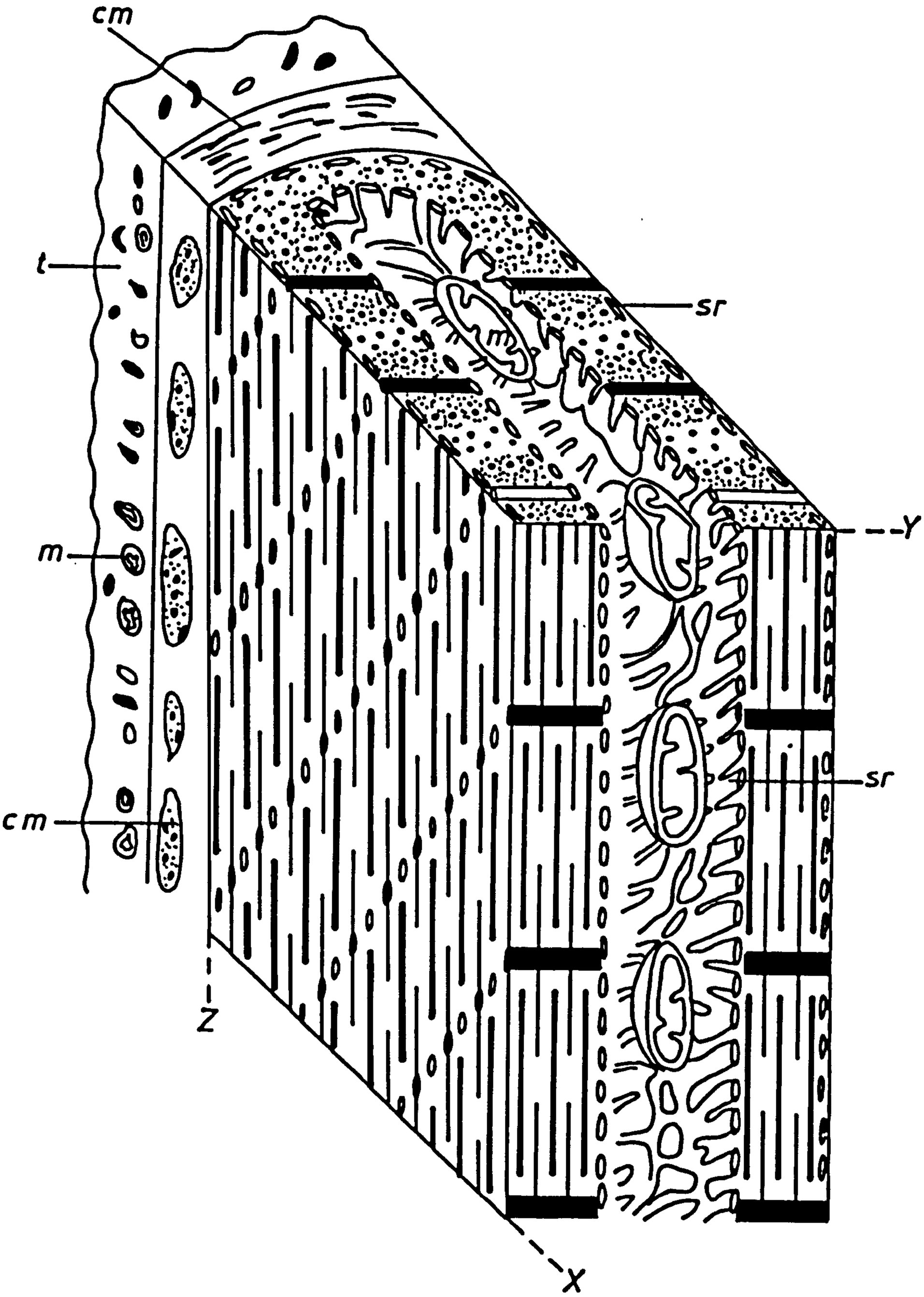
Figure 7.4 Schematic representation of the contractile region of a single striated muscle fibre in the tail of a T. patialense cercaria, demonstrating its three-dimensional organisation.

In the XZ plane the myofilaments are staggered with the result that the striations are slightly oblique rather than transverse.(In the diagram the angle of the striation in this plane has been exaggerated for clarity and the sarcoplasmic reticulum near the sarcolemma has been omitted.)

In the YZ plane the myofilaments are disposed longitudinally while the long axes of Z rods and tubules are in the plane of the section.

In the XY plane the thick and thin myofilaments are sectioned transversely while the long axes of the Z rods and tubules are in the plane of the section.

cm: circular non-striated muscle fibres; m: mitochondrion;
sr: cisternae of sarcoplasmic reticulum; t: tegument


cm
t
m
cm
sr
Y
sr
Z
X

clearly in sections approximating to the YZ and XY planes (Plates 7.4A and 7.5A). It is important to note that most, if not all, the electron micrographs of this chapter show striated muscle fibres in a contracted condition. It appears that the procedures utilised during cercarial fixation bring about this state in the musculature. As a consequence of this situation I bands are usually narrow.

Sections of the myofibres show alternation of light and dark bands (Plate 7.4A and Plate 7.6) formed by the presence of two types of myofilaments, thick ones with a diameter of approximately 22 nm and having tapering ends, and thin myofilaments with a diameter of about 5 nm (Plates 7.5B and 7.7).

Sections in the XZ plane show the A and I bands running at a slight angle to an axis normal to the longitudinal axis of the myofibre (Plate 7.6A), while in the YZ plane this obliqueness is not apparent (see Figure 7.4, Plate 7.4A).

Each dark band (A band) is 0.63 μm - 1.14 μm in width (i.e. parallel to the Z axis). As the sectioned muscles were contracted the A bands contain both thick and thin myofilaments with a central zone containing only thick filaments (H zone) (Plate 7.6A).

The spatial interrelationships of the parallel thick myofilaments within a sarcomere are most strikingly apparent in sections in XY plane (Plate 7.5A). The myofilaments are disposed in rows running roughly normally across the approximately 0.7 μm width of the contractile region. Each such row consists of about eight to 10 evenly spaced myofilaments. The positions of myofilaments in adjacent rows, however, are almost exactly out of register so that the overall arrangement of thick myofilament cross sections in the XY plane becomes a skewed hexagonal packing. Most thick myofilaments viewed in transverse section were circular in profile with a hollow core (Plate 7.5A).

The thin myofilaments within the A band, as seen in sections in the XY plane, can take on a number of spatial arrangements. In some circumstances, no particular pattern is apparent with the thin myofilaments disposed apparently randomly between the thick. Often, however, much more order is observable. In these circumstances, each thick filament is surrounded by a ring

of about 10-12 thin filaments, all approximately equidistant from it. Individual thin filaments, though, are "shared" by adjacent thick filaments so that individual thin filaments may be part of two or three rings (see Plate 7.5B). In the centre of the I band is a conspicuous Z region (Z line) dividing the myofibres into a series of sarcomeres measuring 0.8-1.3 μm along the Z axis (Plate 7.6A). Each Z region consists of an alternating sequence of electron dense rods and tubular elements separated by short spaces (about 8-10nm) (see Figure 7.4). Since these elements correspond to the Z line of other striated muscles, they will be called Z rods and Z tubules (see Jamieson, 1981). In the XZ plane, each rod has a characteristic cross sectional square profile 40-60 nm across, with projections that give it the appearance of an H with a thick crossbar (Plate 7.6A). At higher magnification (Plate 7.6B) the rods can be seen to be made up of amorphous dense material with laterally situated dense threads to which the thin myofilaments are probably attached. The thin myofilaments appear to extend from each lateral part of each dense rod and form the I band on either side of the Z line (see Plate 7.6B).

The Z tubules which arise from cisternal elements of the sarcoplasmic reticulum at the periphery of the myofibres (Plate 7.5C), pass across the myofibre at regular intervals in the middle of the I band, alternating with the Z rods (Plate 7.6A and B). No direct connection between these tubules and the sarcolemma has ever been observed. Each tubule as observed in the XZ plane normally exhibits an ovoid cross sectional profile, in the plane of the Z line with a minimum diameter always of 50 nm and more variable maximum diameter of between 80 and 160 nm (Plate 7.6B). In sections in the YZ and XY planes (see Plates 7.4 and 7.5) the Z rods and tubules appeared to extend all the way from the periphery of the contractile region of a myofibre to its central face.

Images in the XZ plane (see for example Plate 7.6B) often demonstrate a highly ordered correspondence between the spacings of the Z line components and the thick myofilaments on each side of a Z line. The contracted condition of the muscle cells means that the ends of the thick filaments closely approach the Z line. In such circumstances it is often apparent that

the longitudinal axis of every thick filament passes through a Z rod or Z tubule. Longitudinal tubules of the sarcoplasm reticulum/Z tubules system with a diameter similar to that of the Z tubules are seen on infrequent occasions extending between the Z lines of adjacent sarcomeres (Plate 7.7).

The sarcoplasmic reticulum of the striated muscle cells is very prominent consisting mainly of a longitudinally oriented network, distributed at the outer and inner surface of the contractile region of the myofibres. At the periphery of each myofibre flattened and interconnected cisternae of sarcoplasmic reticulum are arranged in close association with the sarcolemma (Plates 7.4B and 7.5C) with some dense material between the two sets of membranes. As mentioned above, the sarcoplasmic reticulum system is directly connected to both the Z and longitudinal tubules.

The non-contractile, nucleated cell body of each striated muscle cell usually extends inwards (i.e. towards the centre of the tail stem) from the contractile region. Oriented in this way the cell bodies contribute along with nervous elements and the excretory duct to the central components of the tail within the muscle layers. Each cell body contains a large ovoid nucleus (approximately 9 μm x 4 μm) in size with prominent nucleolus and clumped chromatin (Plate 7.8). The perinuclear cytoplasm contains aggregations of glycogen granules and a few mitochondria. These two cytoplasmic components also characterise the cytoplasm enclosed by the myofilaments part of the cell (Plates 7.4A and B; 7.6A; 7.10). In the latter regions, however, the mitochondria become larger (about 2.3 μm x 0.71 μm), more numerous and possess high densities of cristae.

(b) Muscle cell arrangements

The cercarial tail is attached to the posterior dorsal surface of the body. The 550 μm x 90 μm cylindrical tail stem bifurcates distally to form two 350 μm long furcae. Each flattened furca increases in width from about 60 μm at its base to 100 μm towards the tip. A unique feature of transversotrematid cercariae is the presence of two 230 μm long arm processes projecting laterally from the proximal region of the tail stem.

Light microscopical observations of living cercariae with phase contrast optics reveal that the tail stem contains four

discrete blocks of striated muscle cells. The blocks are arranged in pairs dorso-laterally and ventro-laterally. These four blocks extend throughout the entire length of the tail stem, but take on different configurations in the arm processes and furcae. The muscle cells from which these blocks are constructed lie obliquely to the longitudinal axis of the tail stem. Each muscle bundle is formed from about 38 long, bipolar muscle cells. In most of these cells, the noncontractile cell body, containing the nucleus, usually bulging into the central lumen of the tail. Anterior and posterior contractile processes are situated on each side of this centrally located nuclear region. The general shape of a striated muscle cell from the central tail stem region is illustrated diagrammatically in Figure 7.5. One unusual feature of many of these cells, particularly those in the central region of the tail stem,concerns the disposition of the anterior and posterior contractile processes. The longitudinal axes of these two processes are displaced dorso-ventrally with respect to one another as shown in Figure 7.5.

The spatial organisation of these striated muscle cells appears to vary in different regions of the tail. For instance, if we divide the tail stem into three parts as shown in Figure 7.6, the following spatial pattern appears to be present:

A- The anterior region has about 10 elongate muscle cells in each bundle. The anterior contractile process of each cell is situated laterally, while the posterior contractile processes are found closer to the midline of the tail stem..

B- The mid region consists of about 16 muscle cells in each block which show a characteristic pronounced V-shape, with the angle of the V containing the nucleus situated centrally with relation to the tail stem.

C- The posterior region consists of about 12 elongate muscle cells in each block which appear similar to the muscle cells in the anterior region except that they are longer and their posterior contractile processes cross over with the similar processes of the contralateral muscle cells. This arrangement produces the region termed "cruciform muscles" by Whitfield, Anderson & Moloney (1975). The cells are inserted posteriorly

Figure 7.5 Diagrams of a striated muscle cell from the mid-region of the tail stem,

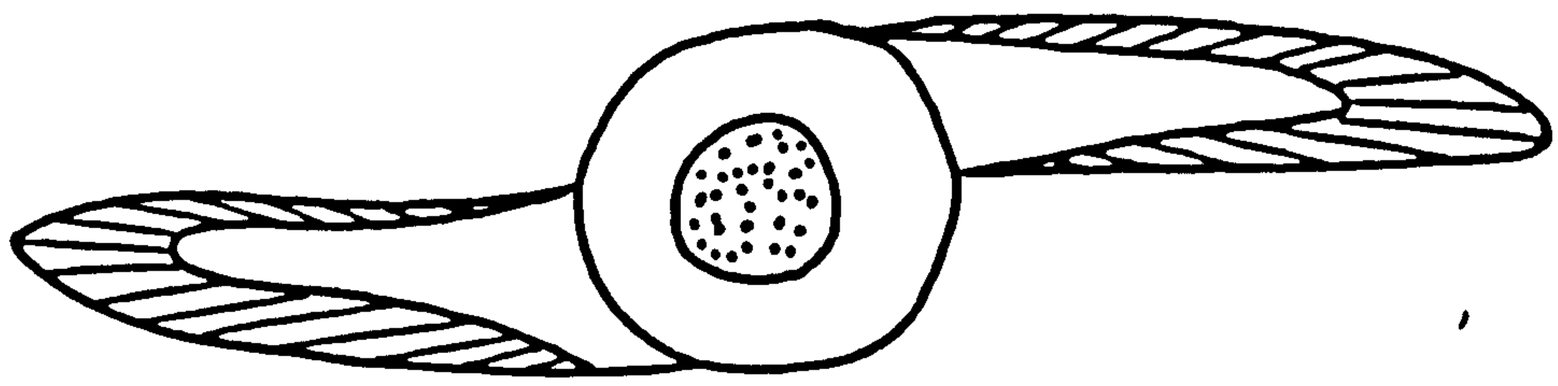
a) viewed from the central axis of the tail stem

b) viewed from the dorsal or ventral aspect

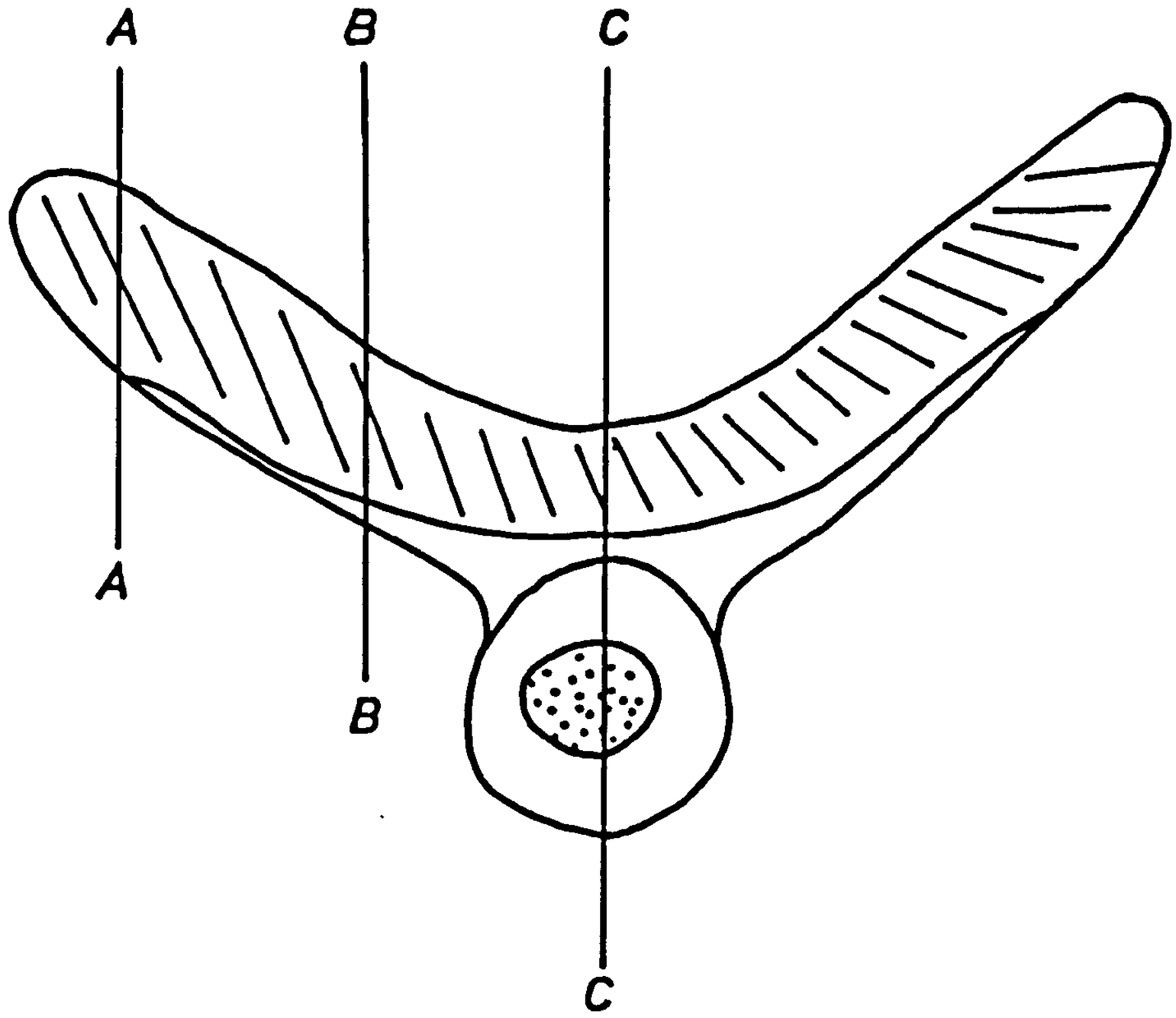
c) transverse section of the muscle cell in different planes

cc shows a transverse appearance seen at the anterior and posterior ends of the tail stem.

a)



b)



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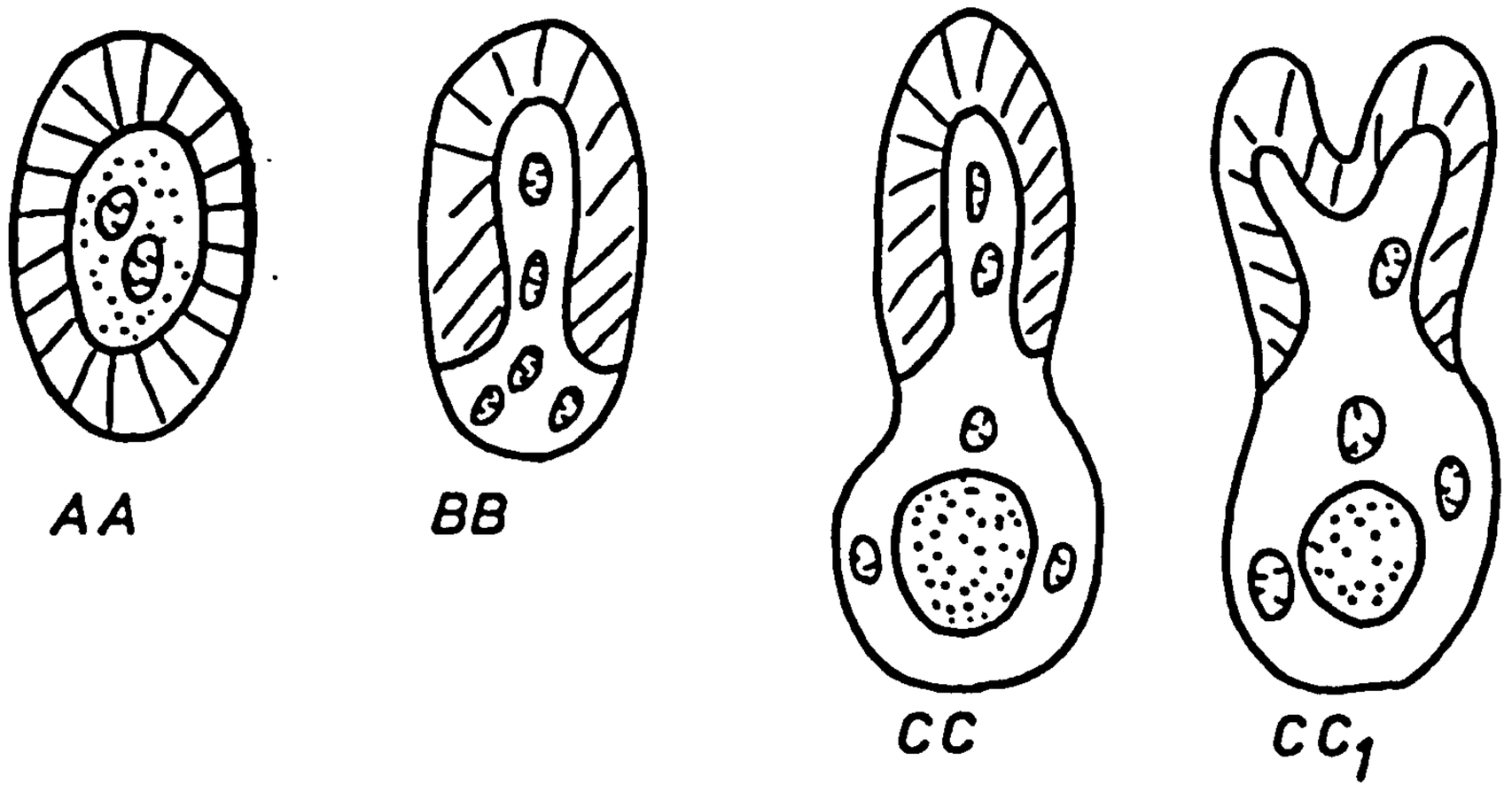
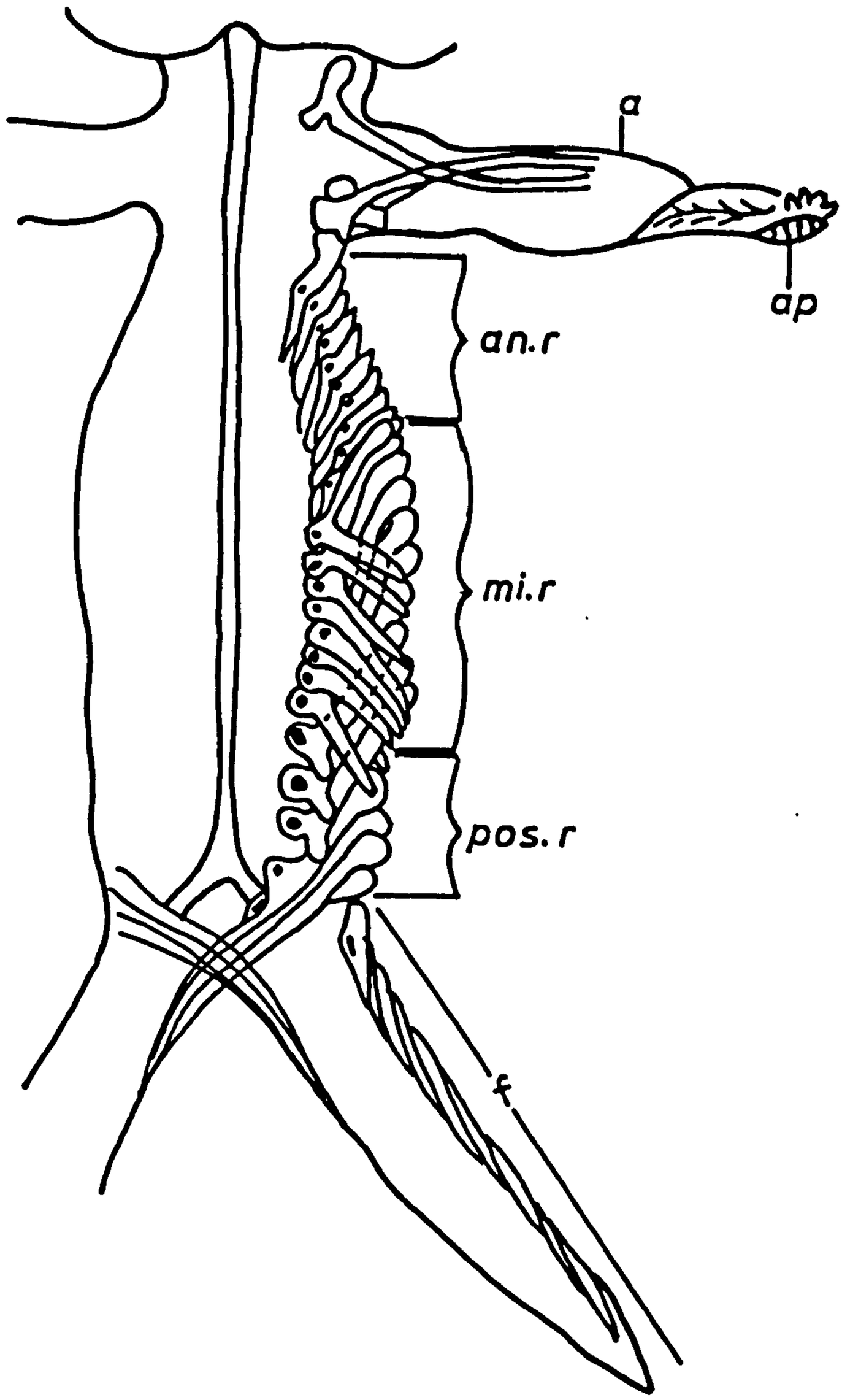


Figure 7.6 Diagram of a T. patialense cercaria showing the arrangement of striated muscle cells from the dorsal side (the number of muscle cells in each region has been reduced for clarity)

a: arm process; an.r: anterior region; ap: adhesive pad; f: furca; mi.r: mid region; pos.r: posterior region


a
ap
an.r
mi.r
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f

onto the medial faces of the two furcae.

Examination of transverse TEM sections in these regions has enabled the general muscle cell patterns observed by optical microscopy to be confirmed and extended. Figures 7.7-7.9 are interpretations of these patterns in the three regions based on such TEM observations. In all regions the tegument of the tail has a broadly similar ultrastructure. The distal cytoplasm of the tegument is approximately 0.81 μm in thickness bounded by a pical and basal plasma membrane.Subjacent to the apical plasma membrane is a 12 nm thick electron dense stratum.
Small oval or round-mitochondia and electron
dense inclusions were found scattered throughout the distal cytoplasm (Plate 7.9). The basal plasma membrane of the distal cytoplasm is attached to an underlying fibrous basal lamina by regularly occurring hemidesmosome, the basal plasma membrane being invaginated at the point of attachment. Occasionally the tips of the tubular invagination become distended giving the appearance of small vesicles. Subjacent to the basal lamina was a layer of interstitial matrix containing fibre approximately 5 nm thick.

Details of the organization and distribution of muscle cells in the three areas of the tail are described area by area below:
A. Anterior region (Plate 7.9): the tail appears approximately circular in transverse section. Underlying the tegument is a thin layer of circular muscle fibres, two blocks of longitudinal non-striated muscle fibres and a sheet of dorso-ventral non-striated muscle cells. Beneath the circular muscle layer the main muscular region of the tail is found, arranged in four blocks of striated muscle fibres.

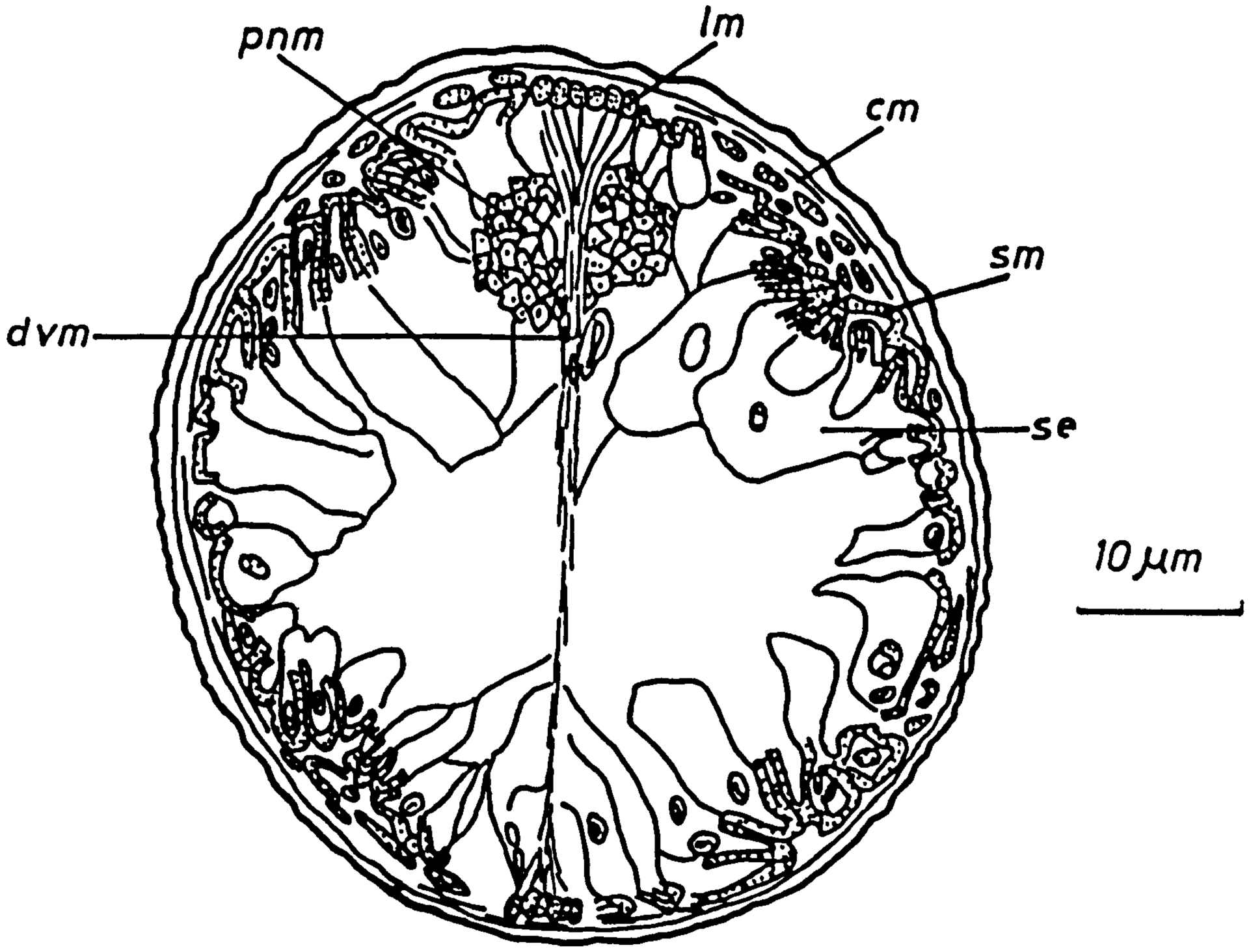
The profiles of the striated muscle cells in these blocks are divided as described above into two types of cytoplasmic regions, a contractile process and sarcoplasmic non-contractile regions. In the more lateral portion of each block, a single layer of contractile process profiles underlie the circular muscle cells. Most dorsally or ventrally the layer of contractile process becomes double with the processes in each layer being at an angle to one another. In these zones the inner set of contractile process is believed to consist of a row of posterior contractile processes while the outer layer is probably composed of the anterior tips of the anterior processes of some "V" shaped

Figure 7.7 Diagrammatic representation of a transverse section of the anterior region of the tail stem.

cm: circular non-striated muscle fibres; dvm: dorso-ventral non-striated muscle fibres; lm: longitudinal non-striated muscle fibres; pnm: proximal nerve mass; se: sarcoplasmic extension; sm: striated muscle fibres; t: surface tegument

Figure 7.8 Diagrammatic representation of a transverse section of the mid region of the tail stem

cm: circular non-striated muscle fibres; dn: dorsal nerve; lm: longitudinal non-striated muscle fibres; se: sarcoplasmic extension; sm: striated muscle fibres; t: surface tegument; vn: ventral nerve


pnm
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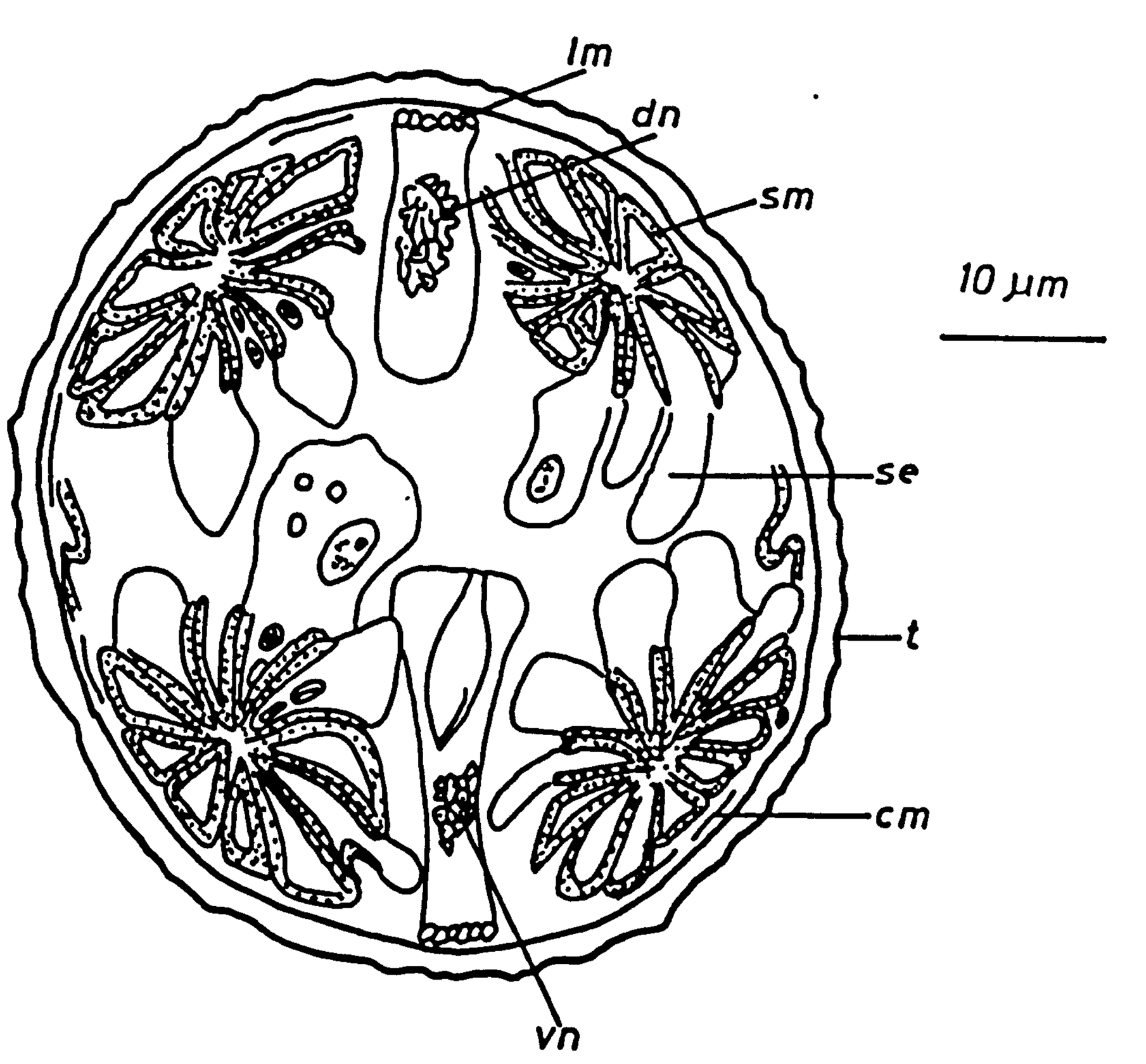
lm
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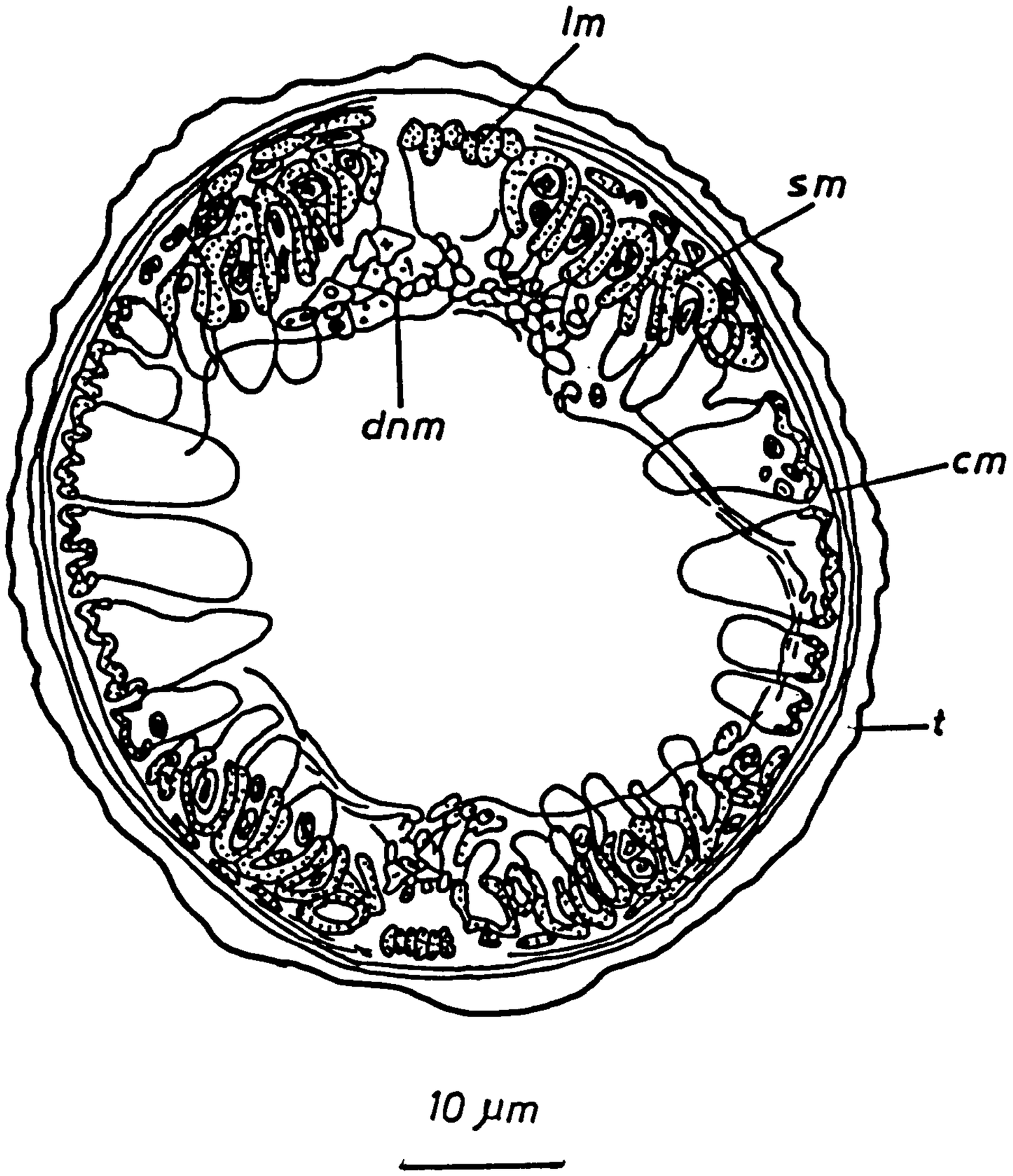
t
cm
vn

Figure 7.9 Diagrammatic representation of a transverse section of the posterior region of the tail stem

cm: circular non-striated muscle fibres; dnm: distal nerve mass; lm: longitudinal non-striated muscle fibres; sm: striated muscle fibres; t: surface tegument



10 µm

cells from the mid region of the tail stem.

The posterior processes can take on two rather different cytoplasmic arrangements. In one (see Plate 7.9, U) the myofibre element is "U" shaped in cross section with the base of the U orientated radially (with respect to the tail stem). In the other, more complex pattern, the myofibre elements takes an "m" shaped configuration with the base of the m pointing inwards with respect to the tail stem. The latter shape (m) appears to be formed by a folding of the "U" shaped system.

Both configurations are "open" on the inner side so that the noncontractile sarcoplasmic region within the contractile process extends inwards to join the nonmyofibrillar nuclear zone which is located centrally.

B. Mid region (Plate 7.10): No dorso-ventral non-striated muscle cells were observed in this region. Circular and longitudinal non-striated muscle cells are very similar in pattern to those of the anterior region.

The disposition of the striated muscle cells in the four symmetrical groups, however, is different from that described from the anterior region. The myofibre regions of the contractile processes appear U shaped in cross section and orientated radially with respect to the tail stem, to produce a "rosette" pattern with anterior and posterior processes very similar in appearance lying one above the other. It appears that the anterior processes lie closer to the tegument than the posterior ones.

The number of "U" shaped profiles in a single block profile ranged between 12 and 16. The rosette pattern itself is due to the "V" shaped configuration of the individual muscle cells overlapping with one another in the manner shown diagrammatically in Figure 7.6. This interlocked pattern is only possible because of the staggered orientation of the anterior and posterior contractile processes.

C. Posterior region (Plate 7.11): The circular and longitudinal non-striated muscle cells of this region are organized in a similar way to those of the anterior and mid regions. The myofibres in the four sets of striated muscles take on several configurations. The most posterior of these cells appear to be almost straight, i.e. not significantly bent into a "V" shape.

In the lateral portion of each block, a single layer of contractile process profiles subtend the circular muscle cells. The processes exhibit a double m (mm) shape in cross section and are probably posterior processes. More dorsally or ventrally the layer of contractile processes becomes double, with the processes in each layer being at an angle to one another. The outer layer seems to be formed from posterior contractile processes which appear as a small solid structure, making close contact with the overlying circular muscle layer.

The inner contractile processes can take on two different cytoplasmic arrangements: "U" and "M" shaped configuration, similar to the configuration found in the anterior region and also oriented radially with respect to the circumference of the tail stem. This inner layer almost certainly represents posterior processes from the last few muscle cells in the mid stem region. The double M (MM) configuration , the posterior process found laterally, are interpreted as the processes which more posteriorly cross over with the similar processes of the contra-lateral muscle cells as shown in Figure 7.6 and Plate 7.13.

All these configurations are "open" on the inner side so that the nonfilamentous sarcoplasmic extensions are directed inwards.The non-contractile system of the myofibres lying at the extreme lateral edges of the muscle block are large, while those of myofibres in the ventral or dorsal portion are somewhat smaller.

7.3.3 Arm musculature

An arm process is a specialised tail structure, projecting laterally from the dorsal surface of the proximal region of the tail stem. It is covered by a tegumentary layer and contains muscles. Each arm process is basically cylindrical in shape, but complicated by two longitudinal, lateral alae which project from its margins. At the distal end of each arm process there is a posteriorly directed ovoid body (adhesive pad) which is a differentiated region of the distal cytoplasm of the epidermal syncytium which invests the whole arm process. The cytoplasm of the pad region contains membrane-bound adhesive granules, which release their contents during activation of the pad (Whitfield etal, 1975). The arm process also bears an array of about nine ciliary sensory structures (the mammiform receptors)

at the distal end and positioned immediately above the adhesive pad.

Three types of muscle cells were found in each arm process: circular non-striated muscle fibres, longitudinal non-striated muscle fibres and striated muscle fibres similar to those of the tail stem. These muscle layers are restricted to the central, non-alar core of each arm process.

The circular non-striated muscle fibres appear as a thin layer closely attached to the inner surface of the basal lamina and surrounding the arm core. The longitudinal non-striated muscle fibres probably extend from the mid dorsal muscle group in the anterior region of the tail. In each arm process these muscle fibres appear to extend alongside the striated muscle blocks in two clusters of fibres.

The striated muscle fibres appear in light microscopic preparations to arise from adjacent regions immediately anterior and posterior to the dorsal point of attachment of the arm process to the tail stem (Figure 7.6) and to cross over near the origin of each arm before traversing an approximately longitudinal course along the arm core.

TEM transverse sections through the central core (Figure 7.10 and Plate 7.13) revealed two asymmetrical blocks of striated muscle fibres. Each block consists of about seven muscle cells with "U" shaped contractile regions.

7.3.4 Furcal musculature

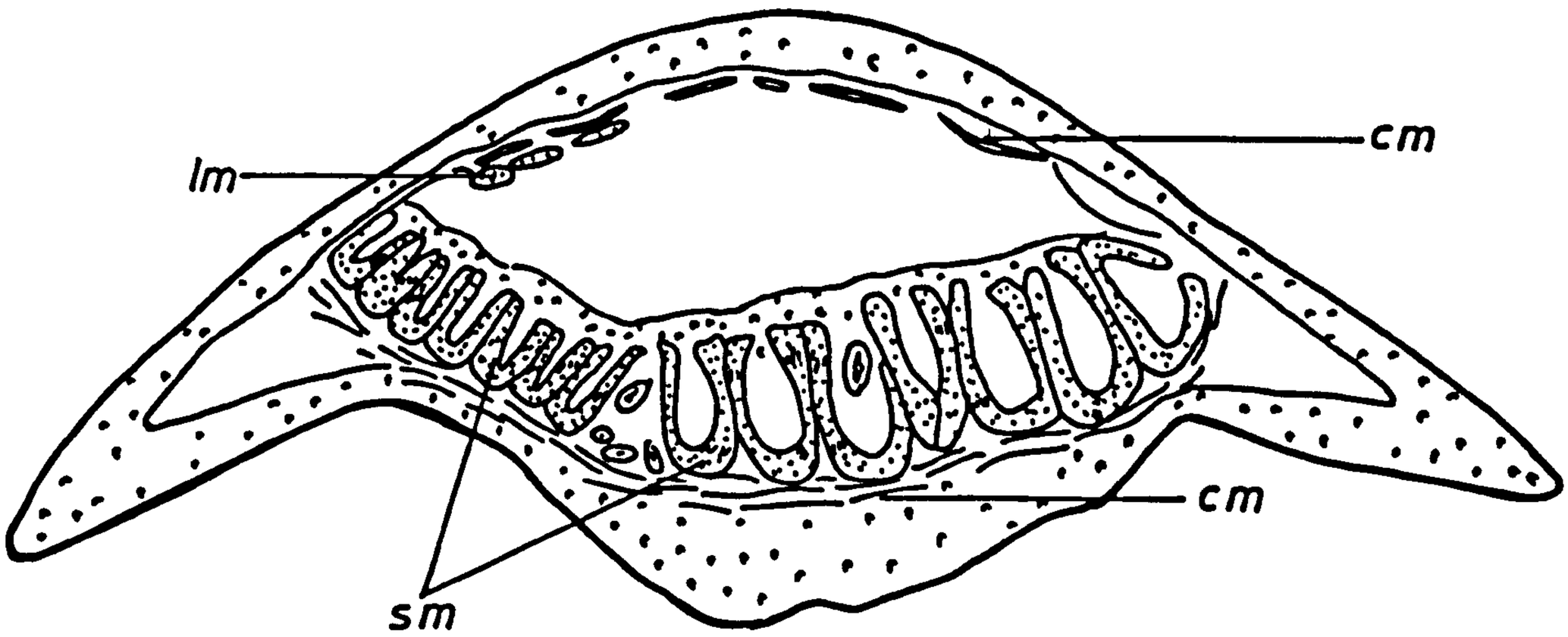
The furcae are long and laterally compressed, and each contains a cavity continuous with that of the tail stem. Four types of muscle fibres were found in each furca:

1. Circular non-striated muscle fibre forming a thin layer lying immediately beneath the basal lamina.
2. Longitudinal non-striated muscle fibres probably continuous with the mid dorsal and mid ventral muscle blocks of the tail stem. These run along the inner medial face of each furca.
3. Striated muscle fibres.

These muscle fibres appear to arise from the tail stem. The posterior contractile processes of the dorso-lateral and ventro-lateral blocks of the striated muscle in the tail stem cross over in the distal region of the tail stem and run along the first third of the medial face of each furca.

Figure 7.10 Section through the arm process of a T. patialense cercaria showing the orientation of the muscle cells in the central core (based on transmission electron micrographs).

cm: circular non-striated muscle fibres; lm: longitudinal non-striated muscle fibres; sm: striated muscle fibres



4 µm

A group of striated muscle arises from the wall of the tail stem laterally on each side and extend along the lateral face of each furca together with other striated muscles attached to the lateral surface with long posterior contractile processes.

Sections of the furca near its proximal end reveal that the striated muscles are arranged along the lateral and medial faces and the longitudinal non-striated muscle fibres restricted to the medial side of the furca only (Plates 7.14 & 7.15).

4. Transverse non-striated muscle cells.

In addition to the previous three types of muscle fibres, there exist narrow transverse fibres which join the lateral and medial surfaces of the furcae and appear to insert into the basal lamina zone under each of the teguments (Plate 7.16).

The tegument in the area of the furcal insertions into the tail stem is highly folded, a feature probably related to the high level of flexing activity going on in this area (Plate 7.12).

7.3.5 Attachment of the tail to the body

At the anterior end of the tail stem the tail narrows considerably and attaches to the posterior margins of the cercarial head. In this junction region considerable modifications of both muscle layers and tegument occur. Most of the important aspects of the ultrastructure of the region are illustrated in Plate 7.17. It reveals that a distinct junctional zone about 12 µm long exists at the extreme anterior end of the tail stem, characterized by an intensely folded tegument. The outer membrane of the distal cytoplasm is highly folded here as is the basal membrane and the fibrous basal lamina beneath it.

Striated muscles terminate immediately behind the junctional zone; they do not pass into either the zone itself or the head. Non-striated longitudinal muscles are very prominent in the zone and appear to represent the anterior branched extensions of the dorsal and ventral muscle blocks of this type in more posterior parts of the tail stem. Anteriorly, these muscles in the junctional zone probably form a continuous muscle layer with longitudinal muscle elements in the body wall of the cercarial head.

The excretory bladder is closely adjacent to the region of fusion between cercarial head and tail. Its posterior margin

(see Plate 7.17B) abuts the anterior portions of the junctional zone. In the central parts of the zone (Plate 7.17B), large diamater longitudinal nerve axons pass between the head and tail.

7.4 Discussion

7.4.1 Body wall musculature

The muscles of the body wall resemble similar muscles described in some turbellarians, monogeneans, digeneans and cestodes (MacRae,1963;1965; Morita, 1965; Lumsden & Byram, 1967; Lumsden & Foor, 1968; Lyons, 1969a; Koie, 1971a; Rees, 1974; Nuttman, 1975).

The myofibres are non-striated, contain parallel thick and thin myofilaments, the latter being continuous with dense bodies. These dense bodies are scattered through the myofibres as is characteristic of muscles which undergo slow contraction. Those attached to the sarcolemma of muscle cells may, as suggested by Lumsden & Foor (1968), be a means for transferring positional changes, generated in the movement of myofilaments to extracellular tissue components for subsequent movement of the worm.

Cisternae of the sarcoplasmic reticulum were present around the periphery of the contractile elements in close proximity to, but not confluent with, the sarcolemma. As has been observed in striated muscles of vertebrates (Franzini-Armstrong & Porter, 1964; Brandt, Ruben & Girardier, 1965; Peachey, 1965), T-tubules connected to the sarcolemma have not, so far, been recorded in platyhelminth smooth muscles.

7.4.2 Caudal musculature

7.4.2.1 Muscle ultrastructure

Cercariae provide a unique opportunity among all the developmental stages and taxa of platyhelminths to examine the complete general range of flat worm muscle types within a single organism. The head and tail possess a complex mixture of non-striated muscle types while striated muscles are present only in the tails of cercariae of some digenean species.

In the tail of the cercaria of T. patialense, the circular, dorso-ventral, mid-dorsal and mid-ventral longitudinal, and furcal transverse muscles all consist of unstriated myofibres of similar general organisation. With their lack of lateral register between adjacent myofilaments, and scattered dense bodies

these muscle cells resemble the smooth, slow-contracting muscles found in most other invertebrates and all vertebrates.

The disposition of dense bodies within the non-striated tail muscles of T. patialense shows one of the wide range of such ultrastructural patterns that exist in smooth muscles in invertebrates and vertebrates.

In vertical muscles of the turbellarian Dugesia dorotocephala (Morita, 1965), for instance, the dense bodies were found to extend across the fibre. For this reason and because of apparent similarity in structure to the Z-lines of vertebrate muscles, Morita named them Z-columns. Some were branched and in close contact with the sarcolemma, and others appeared to anastomose with fibrous structures of the surrounding connective tissue. Z-columns were also associated with tubules of the sarcoplasmic reticulum lying under the sarcolemma. A somewhat similar situation occurs in rabbit arteriole smooth muscle cells where dense bodies form bar-like structures which are regularly distributed and project inwards from the sarcolemma (Rhodin, 1967). Both Morita (1965) and MacRae (1965) compared the dense bodies of invertebrate fibres with the Z-discs of vertebrate fibres. The unstriated caudal musculature of T. patialense does not apparently contain such elongate dense bodies. They are instead tapering ovoids in shape similar to those of mammals (Caesar, Edwards & Ruska, 1957; Pease & Molinari, 1960; Menilleas, Burnstock & Holman, 1963).

The sarcoplasmic reticulum of the T. patialense non-striated muscle cells is relatively sparse. No T-tubules have been observed in cercarial unstriated muscles and so the peripheral disposition of the sarcoplasmic reticulum cisternae beneath the sarcolemma might in some ways be the functional equivalent of the T. system in that sarcolemma depolarizations might be directly able to influence the adjacent sarcoplasmic reticulum cisternae. T-tubules may be few or absent altogether in vertebrate skeletal muscles with very slow contraction and long relaxation times (Peachey & Huxley, 1962; Hess, 1965; Page, 1965).

The fibrous material in the intercellular spaces around T. patialense longitudinal non-striated muscles probably functions as an extracellular skeleton and the attachment of the dense bodies

in some places to this fibrous material via hemidesmosome-like contacts could provide anchorage points for the contractile elements.

The suggestion has been made that the circular muscles of the tail might be anucleate (see Pearson, 1961) but if so, this can not apply to all species as Chapman (1973) and Nuttman (1974) have found such nuclei in association with the circular muscle fibres of Himasthla secunda, Cryptocotyle lingua and Schistosoma mansoni respectively.

In this study, despite repeated attempts to demonstrate such nuclei, no unambiguous links between circular muscle contractile regions and nucleated cytoplasm have been discovered. Rees (1975) was similarly unable to find connections of this sort in the circular muscles of the tail of Cryptocotyle lingua cercariae. This worker suggested that the muscles are probably nucleated but that the cytoplasmic connections between the two regions of the cells must be long and tenuous. It is likely that a similar condition is present in the species under study in this report.

Previous descriptions of the caudal musculature of different species of cercariae have shown that although the same basic layers are always present, the orientation of longitudinal myofibres may vary between species.

In the present study, the longitudinal myofibres in the four main striated muscle blocks were seen by light microscopy of whole mounts to lie at distinct angles to the longitudinal axis of the tail. Most of this angled appearance is due to the grouped sets of anterior and posterior contractile regions of these cells which are at an angle to one another. This angle changes along the length of the tail stem.

The oblique orientation of some of the longitudinal muscle fibres of cercarial tails has also been noted by other workers in a range of digenean species, including Neodiplostomum intermedium (Pearson,1961), Heterobilharzia americana (Lumsden & Foor, 1968) and Schistosoma mansoni (Nuttman, 1974). In each of these cases the obliquely disposed longitudinal muscles were striated. Cardell & Philpott (1960) postulated that the striated longitudinal muscles of Himasthla quissetensis formed a complete layer beneath the circular muscle, with the myofibres running spirally around the tail. Chapman (1973) working on the tail ultrastructure of

a cercaria of another species of the same genus, namely H. secunda found that the longitudinal caudal muscles had a greater cross-sectional area dorso and ventro laterally, to produce four main muscle blocks and that there was no lateral longitudinal muscle at all. He also found this same quadripartite general pattern in Cryptocotyle lingua. Given Chapman's findings, the spatial interpretation of Cardell & Philpott (1960) seems unlikely to be realistic.

Rees (1975) has found that the striated longitudinal muscle cells in Cryptocotyle lingua extend in a straight line along the tail length, and Sandararaman & Nadakal (1979) have come to a similar conclusion with respect to the transversotrematid larva, Cercaria chackai.

The presence of four main dorso- and ventro-lateral longitudinal muscle blocks appears, therefore, to be common to Neodiplostomum intermedium (Pearson, 1961), Himasthla secunda and Cryptocotyle lingua (Chapman, 1973), Schistosoma mansoni (Nuttman, 1974), Cercaria chackai(Sundararaman & Nadakal, 1979) and T. patialense (this study) (see Figure 7.11).

The most remarkable fine structural feature of the cercarial muscle in the four main blocks is their distinct striated organisation. These muscles appear to be the only true striated muscles in platyhelminths.

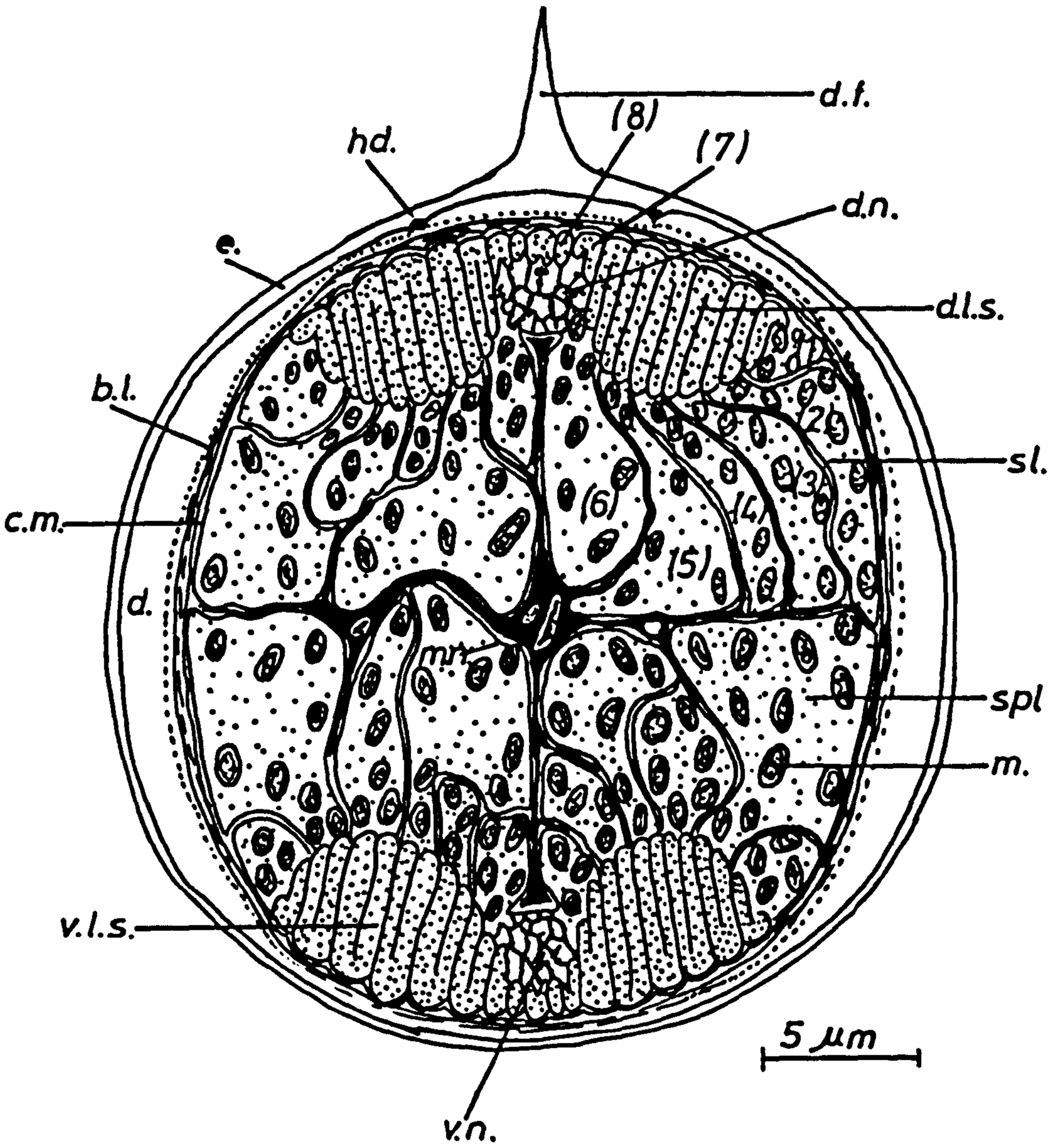
The striated myofibres of cercarial muscle have both thick and thin myofilaments. The latter are attached to Z material and the two types of myofilament are arranged in interdigitating arrays to give rise to A and I bands and an H zone similar to those of vertebrate and invertebrate striated muscle.

The basic organisation of the contractile elements is slightly oblique with characteristic repeated patterns of H,A and I bands and Z line. Components resembling in many ways the obliquely striated muscle of nematodes and annelids (Rosenbluth, 1965a; 1968; Heumann & Zebe, 1967; Mill & Knapp, 1970a). In particular, the Z line areas possess exactly the same alternating sequence of Z rods and Z tubules as has been described for a number of annelid obliquely striated muscles (see Mill & Knapp, 1970a).

Striated muscle in cercarial tails showing some features of classic striated vertebrate muscle (Huxley, 1953; 1957) have been reported by Cardell & Philpott (1960); Lumsden & Foor (1968);

Figure 7.11 Diagrammatic transverse section through the tail of Cryptocotyle lingua, near the origin of the dorsal fin, showing the dorsal and ventral longitudinal muscles and the central multipolar neuron. (From Rees, 1975)

bl: basement lamina; cm: circular muscles; df: dorsal fin; dls: dorsal longitudinal striated muscle cell ((1)-(8)); dn: dorsal nerve; e: outer epidermal layer; hd: hemidesmosome; m: mitochondrion; mn: multipolar neuron; sl: sarcolemma; spl: sarcoplasm of muscle cell; vls: ventral longitudinal striated muscles; vn: ventral nerve


d.f.
(8)
(7)
hd.
d.n.
e.
d.l.s.
b.l.
sl.
c.m.
(6)
(4)
(5)
d.
m.r.
spl
m.
v.l.s.
5 µm
v.n.

Chapman (1973); Nuttman (1974); Rees (1975); Reger (1976); Sundararaman & Nadakel (1979). It is generally accepted that such an organisation is associated with an ability to contract and relax rapidly.

The components of striated muscle which are involved in the mechanism of muscle contraction, namely over-lapping cross-linked thick and thin myofilaments and the Z bands to which the thin myofilaments are attached (Hanson & Huxley, 1953) are all present in the tail of the cercariae of T. patialense. A and I bands, however, were not recognizable in the tail muscles of Himasthla quissetensis (Cardell & Philpott, 1960) and Heterobilharzia americana (Lumsden & Foor, 1968). As during contraction, the thin filaments slide between the thick filaments, resulting in an increase in their overlap. The I band and the H zone both necessarily diminish. Such a narrowing of the I band might be the cause of the failure of Cardell & Philpott (1960) and Lumsden & Foar (1968) to observe I and A bands. In addition, however, the individual layer of thick filaments slide against each other. As a result, the angle at which the bands in the relaxed state are oriented with respect to the longitudinal axis of the contractile region, increases as the muscle contracts.

The thick filaments in the A bands are surrounded by 10-12 thin filaments. An orbital distribution of myofilaments around individual thick myofilaments with cross links between the thick and surrounding thin myofilaments has been demonstrated in vertebrate striated fibres (Huxley, 1957; Franzini-Armstrong & Porter, 1964) and various invertebrate muscle types (Hanson & Lowy, 1961; MacRae, 1963; 1965; Morita, 1965; Rosenbluth, 1965a; Hagopian, 1966; Mill & Knapp, 1970a; Nuttman, 1974; Rees, 1975; Sundararaman & Nadakal, 1979).

The number of thin myofilaments associated with a single thick myofilament varies from six in vertebrate striated fibres and insect flight muscle fibres to 10 and more in other insect muscle types, nematode, annelids and platyhelminth fibres. In some cases this variation may be due to overlapping of myofilaments in highly contracted fibres. The high ratio of thin to thick myofilaments seems to augment the tensile strength of the fibres

and also creates more reactive sites between the thick and thin myofilaments (Hagopian, 1966).

In the present study the thin myofilaments are similar in diameter (5 nm) to the actin filaments in vertebrate cross-striated muscle. The thick or myosin filaments, however, are much greater in diameter (about 22 nm) than those of typical vertebrate muscles (11 nm, Huxley & Hanson, 1954). The thick myofilaments of the striated muscles in the tail of T. patialense appear electron transparent in their centres. This appearance has also been reported in other cercariae (Rees, 1975; Sundararaman & Nadakal, 1979), in non-striated muscles in cestodes (Lumsden & Byram, 1967) and in arthropod muscles (Smith, 1961; Hagopian, 1966).

In T. patialense cercariae, the banded appearance of the muscle fibres is accentuated by the regular spacing of Z lines demarcating sarcomeres. The lines are composed of dense rods (Z rods) and transverse tubules of the sarcoplasmic reticulum (Z tubules) as has also been noted by Lumsden & Foor (1968) in Heterobilharzia americana, Nuttman (1974) in Schistosoma mansoni, Rees (1975) in Cryptocotyle lingua, Reger (1976) in Schistosoma sp and Sundararaman & Nadakal (1979) in Cercaria chackai. Other authors have not described the bipartite nature of the Z lines of cercarial striated muscles noting either the dense bodies alone, e.g. Chapman (1973) in Himasthla secunda and Cryptocotyle lingua, or tubules of sarcoplasmic reticulum alone, e.g. Kruidenier & Vatter (1958). As a working hypothesis it would seem reasonable to suppose that all cercarial striated muscles actually possess Z lines composed of both Z rods and transverse tubules.

Fragmented Z bands have been noted by North (1963) in the cardiac muscle of Helix aspersa, Hanson & Lowy (1961) in the adductor muscle of the oyster Crassostrea angulata, and Hoyle & Mclear (1963) in the muscles of the barnacle Balanus nutilus who found that during contraction, the thick myofilaments pass through the spaces between the bars, a condition not observed in the cercariae of T. patialense. It is probable that these dense rods are the functional equivalent of the Z disc of vertebrate muscle. The Z disc is considered to be important in the coordination of myofilament movement (Franzini-Armstrong & Porter, 1964).

The striated muscle of T. patialense cercariae displayed a

well developed sarcoplasmic reticulum, a feature commonly associated with fast acting muscles. This reticulum is an elaborate system of tubular and cisternal structures associated with myofibres. The transverse tubules of sarcoplasmic reticulum extending across the myofibres alternating with the dense rods of the Z lines in T. patialense are not present in vertebrate striated muscle but have been noted in Heterobilharzia americana (Lumsden & Foor, 1968); Schistosoma mansoni (Nuttman, 1974); Cryptocotyle lingua (Rees, 1975); Schistosoma sp. (Reger, 1976) and Cercaria chacki (Sundararaman & Nadakal, 1979).

The transverse T. tubule system of vertebrate striated muscle (Porter & Palade, 1957; Porter, 1961; Revel, 1962) is absent in the striated muscles of T. patialense cercariae. The transverse Z tubules are not confluent with the sarcolemma.

This pattern of sarcoplasmic reticulum organisation poses interesting functional questions relating to T. patialense striated muscles. Specifically, which parts of the system are concerned with the storage and release of Ca^{++} ions, the apparent role of all such systems, and which regions, if any, are concerned with a propagation of excitation to deep regions of the cell.

T. tubules do, however, occur in the striated muscles of some invertebrates. Fahrenbach (1963) and Rosenbluth (1965a) have, for instance, described them in the muscles of Macrocyclops albidus and Ascaris lumbricoides respectively.

Constantin, Franzini-Armstrong & Podolsky (1965) have demonstrated that the terminal cisternae of the sarcoplasmic reticular system accumulate calcium which may, on depolarization of the adjacent surface membranes of the cisternae and sarcolemma, be released from the cisternae to the myofilaments where it is necessary for contraction. The electrophysiological investigations of Huxley (1957) and Huxley & Taylor (1958) on crab, frog and lizard muscle demonstrated that the excitation is carried into the muscle fibre preferentially at those levels at which the transverse T. tubules are located.

With no T-tubule system connected to the sarcolemma in the T. patialense cercariae striated muscles, conduction of excitation into the cell must be by indirect means. The flattened sarcoplasmic cisternae exist as two fenestrated sheets, one immediately beneath the sarcolemma, the other about 0.7 μm

deeper into the cell adjacent to the mitochondrial core of the contractile region. It is concernable that the outer sheet of cisternae is close enough to the sarcolemma to be activated where ever the adjacent regions of sarcolemma are depolarized due to actions at a nerve-muscle junction elsewhere on the cell surface. Spatial considerations, however, suggest that the inner fenestrated layer is unlikely to be susceptible to such stimulations, distanced as it is from the sarcolemma by the thickness of the sarcomeric zone of the contractile regions.

Viewed in this context, the Z-tubules, linking as they do, the two sarcoplasmic reticulum sheets, might well be supposed to have a propagatory role in terms of excitation. If, when the outer sheet is activated, a propagated change in permeability to Ca++ ions passes inwards along all the Z-tubules this change could stimulate Ca++ release by the cisternae of the inner sheet. Such an arrangement could ensure that Ca++ ions were available for myosin-actin interactions from both sides of the sarcomeric layer more or less simultaneously.

The longitudinal tubules that this study has shown to directly link the transverse tubules of one Z line with those of adjacent ones, could aid in the transmission of the permeability change throughout the cell. Interestingly, intrafilamentous sarcoplasmic reticulum elements, similar to the longitudinal tubules, have also been observed in other fast-acting invertebrate muscle. For example, Macrocyclops albinus (Fahrenbach, 1963); Achalarus lyciades (Reger & Cooper, 1967); Cercaria chackai (Sundararaman & Nadakal, 1979); Limulus polyphemus (Eagles & Riodan, 1980).

The presence of large and numerous mitochondria in T. patialense striated muscles within the contractile region core must be correlated with high metabolic activity. The abundance of glycogen particles close to the mitochondria and within the nucleated region of each muscle cell is also presumably associated with the high energetic requirements during swimming. The glycogen decreases gradually in quantity with increasing cercarial age (Anderson & Whitfield, 1975).

7.4.2.2 Muscle function

The results reported in this chapter have demonstrated that the tail of the cercaria of T. patialense possesses an extensive and diverse set of muscles. In attempting to understand the separate

and interrelated functional roles of these muscles, it is probably useful to itemize the range of tail-related phenomena that are likely to the mediated wholly or partly by muscular activity. The following list is probably not complete, but contains all the important processes yet identified.

(a) Rapid lateral bending of the tail stem

T. patialense cercariae swim in a tail-first manner using alternate lateral bendings of the tail stem in which the furcae act as thrust-producing vanes. A notable feature of this activity is its very high frequency (up to 30 complete wave forms per second) (Whitfield etal., 1975; Bundy, 1979, 1981a). The muscle which generates this bending activity must possess a number of intriguing properties. Assuming as is likely that one set of muscles bends the tail in one direction, while an antagonistic set bends it in the other, each set must be able to achieve a full contraction in the very short period of approximately 15 milliseconds. Equally, each set of muscles must be ready to contract once again about 30 milliseconds after the commencement of a contraction. Such rapid contraction/elongation cycle times are characteristic of striated fast muscle fibres. The only candidates in the tail of T. patialense cercariae for this role are the obliquely disposed striated longitudinal muscle blocks.

As discussed above, they have the ultrastructural features of rapidly contracting muscle. They are also the most massive of the muscles in the tail and they are spatially organized in two potentially antagonistic sets. Each set is represented by the two blocks in each lateral half of the tail stem. Contraction of one of these sets might be expected to bend the stem in one lateral direction, contraction of the other to bend it in the opposite sense. It is interesting that the contraction of one set necessarily extends the fibres of its contralateral partners.

If these muscles do provide the main propulsive power for tail-first swimming, they are accomplishing what is undoubtedly the most energetically demanding of the activities of the cercariae. In this context it is not surprising that it is within these muscles that the bulk of the tails glycogen reserves are to be found.

(b) Slow movements (scissoring) of furcae

Immobile "resting cercariae", lying at the bottom of a container can show slow bilateral movements of the furcae. These scissoring movements involve the two furcae extending simultaneously towards the longitudinal axis of the tail and then flexing once again.

The slow time course of furcal scissoring activity (the movement from the fully flexed to the fully extended configuration may take up to one second) suggests immediately that it is achieved by slow-contracting muscle fibres. This piece of evidence alone points to the unlikelihood of the striated muscles of the furcae being implicated in these movements. The same evidence raises the possibility that non-striated muscles are, in fact, involved. The only non-striated muscles which are positioned in such a way as to produce "scissoring" movements are the non-striated longitudinal muscles that are extensions of the dorsal and ventral longitudinal muscles of the tail stem. These extensions pass from the stem on to the inner surface of the medial face tegument of the furcae. If the furcae are relatively rigid objects due perhaps to the presence of a hydrostatic skeleton, contraction of the non-striated longitudinal muscles would be likely to bring about slow extension of the furcae. Relaxation of those same furcal muscles would allow the postulated hydrostatic skeleton to return the furcae to a flexed configuration.

(c) Head/tail flexing

Whitfield et.al, 1975 and Bundy (1981a) have shown that the swimming, dropping and resting phases of the behaviour of T. patialense cercariae are all carried out while the cercarial body is in a flexed attitude with the ventral surface of the head in contact with the ventral aspect of the tail stem. Indeed, apart from the behaviour which occurs on the surface of a fish host, T. patialense cercariae are always in this configuration. No direct evidence concerning the muscles which bring about this flexture has been gathered in the present study. From the position of the flexture, though, it seems probable that the initiation of the flexed configuration must be due to an asymmetrical contraction of non-striated longitudinal muscles in the junctional region.

Contraction of the muscles on the ventral aspect of this region could bring about the observed orientation of the head with respect to the tail. Once in this position, it is likely (Whitfield, et al. 1975) that the ventral sucker adheres to the tail stem, "locking" the animal in this flexed shape.

(d) Arm process movements

The arm processes are involved in the initial attachment of cercariae to their fish hosts (Whitfield et.al.1975) and seem to engage in at least two forms of activity. They move in a coordinated fashion during swimming and probably bend during the movements relative to attachment and body inversion after attachment.

During swimming activity an arm process extends laterally from the cercarial head on the side of the cercaria away from which the tail stem is moving at that moment, while the other arm process is pressed against the head circumference which oscillates about the path of movement of the cercaria in sympathy with the tail stem oscillation (Bundy, 1979, 1981a). These movements of the arms during swimming have the same periodicity as the tail stem bending movements. Using the same type of functional arguments as were used above in respect of the relationship between swimming and the striated muscles of the tail stem, it appears probable that the rapid movements of the arms are the result of the contractions of their own longitudinal striated muscle sets. These extend from the blocks in the tail stem and it is easy to imagine ways in which stem and arm movements could be coordinated. The two sets of striated muscles within each arm probably act in an antagonistic fashion towards one another enabling the outward and inward movements with respect to the head circumference to be achieved.

The more complex movements of the arms after attachment to a fish (Whitfield et.al,1975) are neither fast nor synchronized in an obvious way with the activity of the striated muscles of the tail stem. In fact during the crucial phases of inversion and head attachment via the ventral sucker the tail stem does not engage in lateral bending. This might mean that these later activities of the arms are the results of the contraction of the non-striated arm muscles.

(e) Head/tail separation and wound closure

Although the details of the processes are unclear it seems likely that parts of both head/tail separation and head wound closure are achieved by muscular contractions.

The prime functions of the tail are locomotion and the initial attachment to a fish host. After more secure attachment by the ventral sucker has been accomplished the tail is lost. Tail loss is fundamentally the result of a mechanical break at the head/tail junction. It occurs during a period of rapid "swimming" activity by the tail with the head firmly attached to the fish. The tail activity will exert a pull on the junctional region but it is unlikely that tail bending alone can cause the breakage. Presumably other changes must simultaneously be occuring in the junctional region to make it mechanically weaker. A number of potential changes are biologically reasonable. A strong contraction of longitudinal non-striated muscles at the rear of the cercarial head at the same time as the similar muscles in the tail stem were contracting might pull apart previously overlapping muscle fibres in the junctional zone. Equally a strong contraction of circular muscles in the junctional region could produce a marked narrowing in this area. Both these muscle contraction patterns could produce a weakening in the crucial zone so that when tail beating re-commences the tail would be pulled away from the head. Strong circular muscle contraction could then aid closure of the wound at the head side in a "purse string" fashion, probably enabling the thick and folded tegumental distal cytoplasm in this region to fuse once again to a contracted cytoplasmic layer.

(f) Furcal flattening

Postural muscle activity may be involved in the maintenance of the flattened configuration of the two terminal furcae of the tail. The small non-striated transverse muscles of the furcae are ideally positioned to carry out this role.

(g) Furcal attitude

During active swimming there is a requirement to hold either one furca at a relatively constant angle to the region of the tail

stem to which it is attached. Muscle activity is likely to be concerned in fulfilling this requirement. Both the medial and lateral sides of the furcae are supplied with a layer of striated longitudinal muscle fibres at least partly originated in the posterior region of the tail stem. Those on the medial faces form the cruciform muscle arrangement. Rapid antagonistic activity of the muscles seems likely to be responsible for bracing a furca while it is the propulsive vane during half of each swimming wave cycle.

(h) Tail hydrostatic skeleton

Much of the central core of the tail stem, arm processes and furcae appears to act as a fluid filled cavity. It is likely that muscles operating in the more solid tissues enclosing this core can exert forces upon it to alter its internal pressure. Such changes could have significance if the fluid core was operative as a hydrostatic skeleton in for instance swimming activity.

It is interesting that each arm process, each furca and the tail stem itself are provided separately with a pair of sets of striated musculature which can presumably antagonize one another. Such antagonism will be aided if both muscle sets in a pair were operating against the constraints of a hydrostatic skeleton. In swimming activity the tail stem, furcae and arms appear to be turgid, stiff structures. It is probable that this stiffness, with its potential for recoil return to a rest position, is a consequence of contraction of both circular and longitudinal non-striated muscles causing a high internal pressure.

CHAPTER 8

The organization of the nervous system of Transversotrema patialense cercariae: Ultrastructural studies

8.1 Introduction

In most digenean life cycles it is the cercarial developmental stage which spends the longest period as a free-living animal in the external environment. Cercarial periods of survival in aquatic habitats are almost invariably longer than those of the other transiently free-living stages, the miracidia. The life cycle of Transversotrema patialense provides a typical example of this generalization; the cercariae have a maximum life span of about 44 hours at 24°C and in a light intensity of 150 lux (Anderson & Whitfield, 1975). The miracidia, in contrast, only survive for up to eight hours (Bundy, 1981b). During their existence outside of hosts, cercariae exhibit a wide range of activities related to locomotion and also to the behaviour relevant to the establishment of contact with the next host in the life cycle, be it a second intermediate host or final host. The effector systems that are implicated in the behavioural repertoire of cercariae are muscles and, to a lesser extent, glands. The coordination and control of the activities of these effectors must be assumed to be partly or largely a function of the nervous system of these larvae. Despite this presumed central role in the life of cercariae, the nervous system of such larval stages has rarely been investigated in detail.

Cercarial receptors have been studied at the ultrastructural level in a variety of digenean species, e.g. Fasciola hepatica (see Dixon & Mercer, 1965); Himasthla secunda (see Chapman & Wilson, 1970); Diplostomum phoxini (see Bibby & Rees, 1971); Zoogonoides viviparus (see Køie, 1971); Paryphostomum segregatum and Echinostoma paraensei (see Matricon-Gondran, 1971); Schistosoma mansoni (see Morris, 1971 and Nuttman, 1971); Transversotrema patialense (see Whitfield, Anderson & Moloney, 1975, and Bundy, 1979).

In addition to these studies on cercarial sensory organization, a few investigations have been carried out on the general ultrastructure of the cercarial nervous system, namely those of Dixon & Mercer (1965) on Fasciola hepatica; Rees (1967) on Parorchis acanthus; James (1975) on Cercaria linearis and Cercaria stunkardi; Nuttman (1975) on Schistosoma mansoni and Rees (1975) on Cryptocotyle lingua.

The aim of the present investigation has been to describe the ultrastructure and routes of nervous elements in both the head and tail of T. patialense cercariae. Attempts have also

been made to correlate these findings on nervous organization with the previous results on regross organization of the n.s. (Chapter 5), the ultrastructure of cercarial muscles (Chapter 7) and neuromuscular pharmacology (Chapter 6) to provide a composite view of the functioning of this larval stage.

8.2 Materials and Methods

The cercariae were collected, fixed and prepared for transmission electron microscopy as described in Chapter 2 (2.5). In an attempt to localise neurosecretory cells at the light microscopical level, cercariae were fixed in Bouin's solution and after dehydration in alcohol and xylene, they were embedded in paraffin wax (M.p.58°C) and 7 µm sections were cut. Separate sections were stained using the aldehyde-fuchsin method of Cameron & Steele (1959) and the chrome haematoxylin-phloxin method described by Bargmann (1949).

8.3 Results

8.3.1 General organization of the nervous system of T. patialense cercariae.

The analysis of the nervous system in the head region of cercariae of T. patialense (or the adult which develops from it) pose some problems because of the unusual shape of the body in this family of digeneans. In more typical elongate digeneans, the nervous system consists of two cerebral ganglia and three or four pairs of anterior and posterior nerves connected by transverse commissures (Bullock & Horridge, 1965). This pattern has become highly altered in the transversotrematids in parallel with the evolutionary transformation of the normal elongate digenean body to one which is (i) broader than it is long, and (ii) has a mouth situated on the ventral surface. Consequently, it is sometimes difficult to analogize components of the nervous system of T. patialense with regions of the more typical system in other digenean .

The morphology of the nervous system of cercariae of T. patialense as seen with the light microscope (Chapter 5) consists of a pair of laterally situated longitudinal nerve tracts each with an anterior thickening. The latter pass anterio-medially and probably represent the two cerebral ganglia. The thickened portions are connected anteriorly by a narrow transverse commissure which curves anterior to the oral sucker. Posteriorly, the two

main longitudinal tracts are also joined near the body-tail junction. One branch arises medially from each presumed cerebral ganglion area and extends backwards to form an internal nerve plexus situated posterior to the oral sucker thus forming a portion of a circumoesophageal nerve ring. Thereafter, these internal nerve branches continue posteriorly to surround the ventral sucker and pass posteriorly joining the posterior link between the two longitudinal tracts. Two pairs of transverse commissures join the inner branches with the main longitudinal tracts. On each side of the worm eight outer branches originate from the two longitudinal tracts and are directed anteriorly, laterally and posteriorly with most of them breaking up into fine branches peripheral to the main tract.

At the body-tail junction there exists nervous mass from which dorsal and ventral median nerve cords extend the length of the tail stem. They are joined at the proximal end of the tail by a dorso-ventrally oriented commissure and distally they end in a bilobed, bilaterally symmetrical nerve mass from each side of which two nerve cords run into the corresponding furca.

The light microscopical studies, particularly those relying on the histochemical demonstration of either acetylcholinesterase activity or biogenic amine localization have failed to reveal the unambiguous presence of nerves passing into the arm processes of the tail. There are reasons, however, for concluding that some nervous elements, at least, must enter these processes. One piece of evidence that suggests this is so, is the presence of a cluster of receptors at the tip of each arm (Whitfield etal., 1975).

In 7 µm sections of cercariae stained with either aldehyde-fuchsin or chrome-haematoxylin-phloxin methods for the demonstration of possible neurosecretory cells, no categorical evidence of significant cellular staining has ever been obtained in this study.

8.3.2 The Ultrastructure of the nervous system of T. patialense cercariae,

TEM sections through the body of T. patialense cercariae reveal that each presumed cerebral ganglion region consists of a central mass of closely packed, unmyelinated axons, the neuropile, surrounded by a layer containing many nucleated cell bodies, the cell rind (Plate 8.1). At least two types of cell bodies can be

distinguished around the periphery of the neuropile (Figure 8.1; Plate 8.1). The most common type (Type 1A) has a large nucleus which exhibits a prominent nucleolus and chromatin network. The perinuclear cytoplasm in these cells is sparse and is characterised by a high density of free ribosomes. The cytoplasm also contains mitochondria and a small amount of rough endoplasmic reticulum. A proportion of the cells which in other respects have a cytoplasmic organisation of this type also possess accumulations of medium-diameter dense-cored inclusions (see below) 45-175 nm in diameter among the ribosomes (Plate 8.1). These will be referred to as Type 1B cells. It is possible that the two cytoplasmic appearance are developmentally related.

A second cellular type (Type II) in the layer of nucleated cell bodies around the central nervous system neuropile contains prominent cisternae of granular endoplasmic reticulum (Figure 8.1; Plate 8.1). The cisternae usually contain fine granular material of low electron opacity. Occasionally, small numbers of large diameter dense inclusions (see below) are also present in the cytoplasm.

Cell processes from the rind cells presumably pass inwards and form a compact neuropile in which synapses are abundant and which constitutes the core of each ganglion, the transverse commissure and the main nerve cords. The proximal and distal caudal nervous masses are largely axonal in content, with very few neural cell bodies being identifiable.

The closely packed nerve processes of the proximal nerve mass extend between the mid dorsal and mid ventral muscle blocks at the proximal end of the tail stem (Plate 8.2A). In contrast, the bilobed distal nervous mass lies beneath the striated muscle layer at the furcal end of the tail (Plate 8.2B).

The longitudinal nerve cords in the body and in the tail of the cercariae are composed of bundles of non-myelinated nerve processes with numerous synaptic junctions. They are seen as large numbers of closely packed profiles of various sizes and shapes. In all sections of nerve processes whether in the nerve cords or the ganglia, several types of inclusions, small mitochondria and neurotubules have been observed.

Two reasonably distinct forms of nervous process occur in

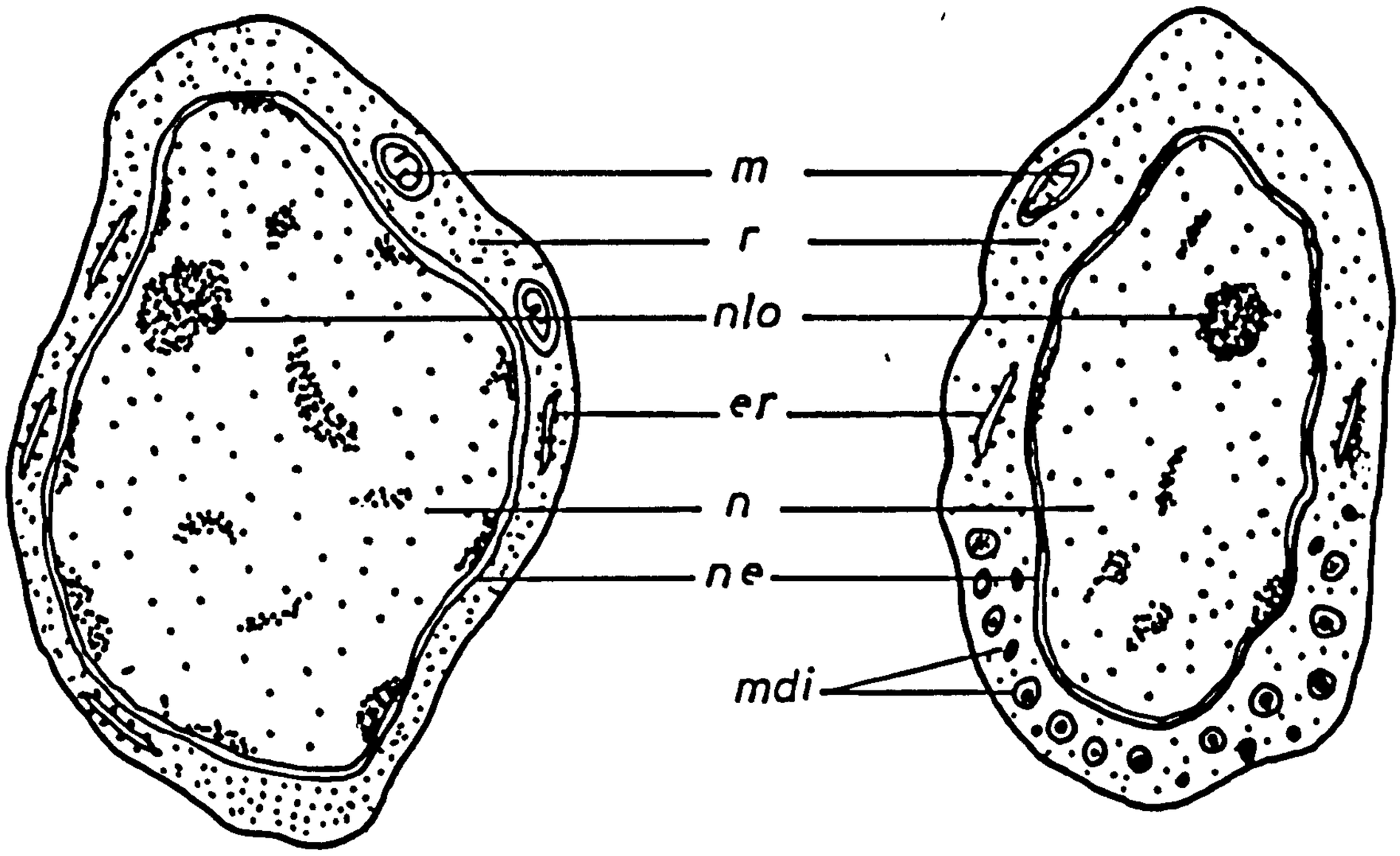
Figure 8.1 Diagram showing the types of nerve cell bodies associated with the neuropile of the cerebral ganglia of T. patialense cercariae

a) Type 1A
b) Type 1B
c) Type 2

er: granular endoplasmic reticulum; ldi: large diameter dense inclusion; m: mitochondria; mdi: medium diameter dense_cored inclusions; n: nucleus; ne: nuclear envelope; nlo nucleolus

a)

b)



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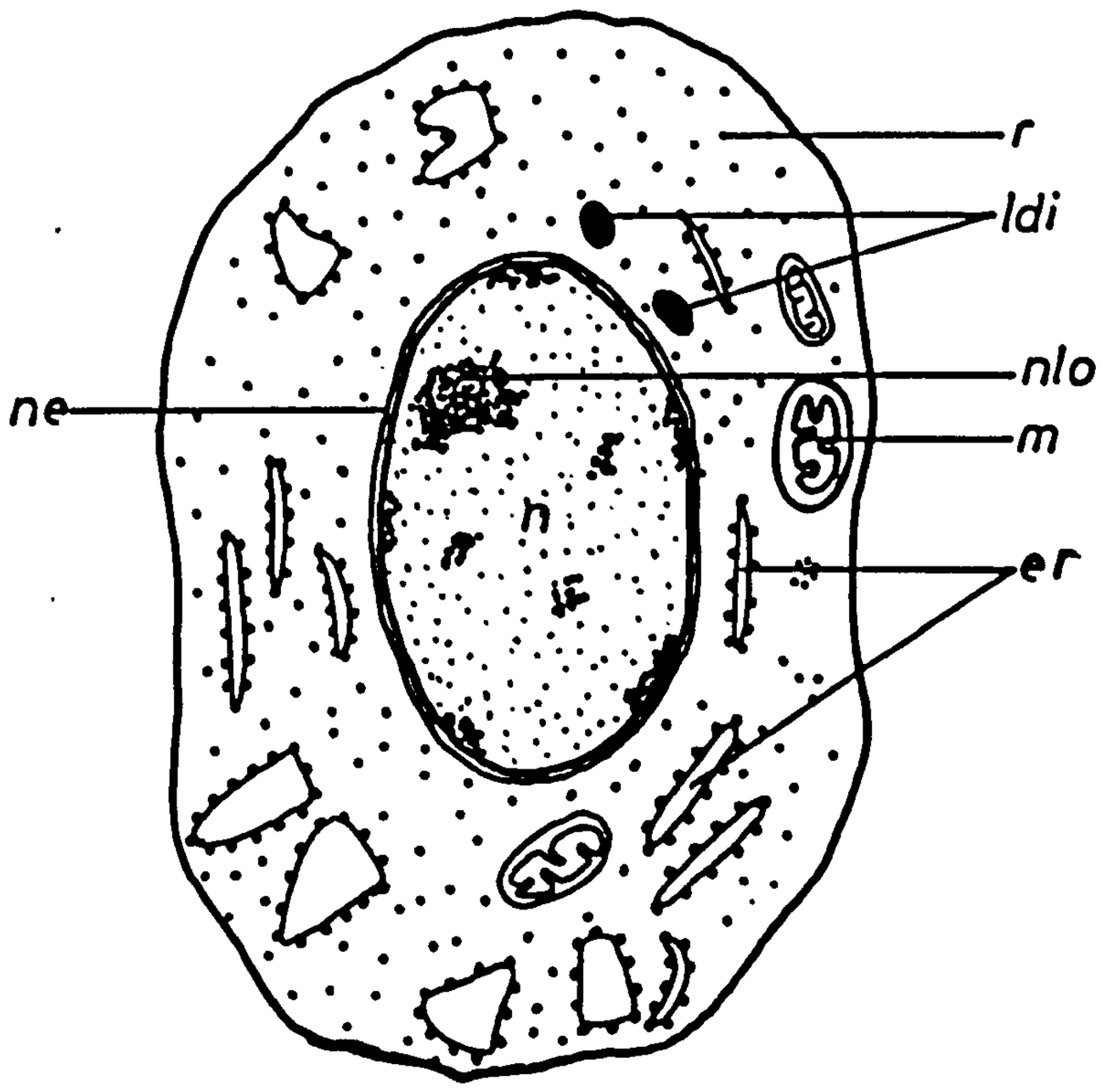


these situations. The first are relatively wide diameter processes (axons) which tend to pass in straight or only slightly undulating courses parallel to the longitudinal axis of the nerve cord. These axons can be as much as (1.82 μm) in diameter but range down to about 0.34 μm (Plate 8.3A). They are further identifiable by their extremely electron lucid contents. In most, the cytoplasm appears "empty" apart from a central longitudinal strand of dense material containing mitochondria, some inclusions and neurotubules. The remainder of the nervous elements of the neuropile consist of smaller diameter processes (dendrites) between 0.22 and 0.61 μm in diameter. These are much more electron dense in their cytoplasmic contents and contain regions packed with inclusions. (Plate 8.3A).

Scattered between the nervous components of the neuropile are narrow strands of non-striated muscle fibres (Plate 8.1, 8.3B). Most of these neuropile associated muscle processes are to be found in the peripheral parts of any neuropile profile and are separated from the nerve processes around them by an extracellular sheath of fine fibres.

As mentioned above, both electron-lucid axons and the denser dendrites can both contain inclusion bodies. Such inclusions can be differentiated into a number of types:

8.3.2.1 Medium-diameter, dense-cored inclusions

Profiles of these inclusions range in diameter from 45-175 nm and are characterised by a range of internal organisations, although almost all reveal a relatively electron-lucid gap between the boundering membrane and the inclusion contents. Some have cores of moderate density, variable size and of homogeneous appearance, whereas in others a coarser, punctate form of core is apparent. The mixture of appearances of these different types in close packed aggregations of inclusions suggests that such polymorphism might be related to inclusions transforming from one form into others within the same general category (Plates 8.3B and 8.4A).

8.3.2.2 Large-diameter, dense inclusions

Lower numbers of these inclusions are found. They are between 110 and 210 nm in diameter, sometimes lack a peripheral gap and possess dense amorphous contents (Plates 8.4B and 8.5B).

8.3.2.3 Small clear vesicles

The most numerous inclusions are small clear vesicles (23-66 nm in

diameter) with a boundring membrane and fine amorphous contents of low electron density (Plates 8.3B and 8.6A).

The three major types of inclusions have somewhat different distributions among the nervous processes. The small clear vesicles tend to occur together in dense accumulations, probably of many hundreds of vesicles (Plate 8.5B). These accumulations are particularly common near nerve-nerve synapses and neuromuscular junctions in head and tail but also occur elsewhere particularly in dendrites. They appear to be uncommon or absent in the large diameter axons. Medium-diameter, dense-cored inclusions are of almost ubiquitous appearance in both axons and dendrites in the head and tail and closely adjacent to synapses. The large diameter dense inclusions, in contrast, seem to be restricted to small diameter dendrites within the head.

Specialised synaptic structures are common in all parts of the nervous system and for the purposes of this chapter it has been assumed that all regions of nerve/nerve contact showing the following two features are likely to be synaptic in character.

a) a close apposition of parallel regions of the cell membranes of two cells,
b) a markedly unbalanced population of inclusions on the two sides of the region of membrane contact.

A range of organisations showing these characteristics has been observed. In many examples of synapses that have been found both the pre- and post-synaptic membranes show deposits of electron-dense material and the synaptic cleft itself is 20-30 nm wide containing dense material which in some cases can be concentrated in the middle of the cleft, so that a dense line is seen along its length in the section. In addition, a variety of dense structures have been observed subtending the post-synaptic membrane (Plate 8.5A,B and 8.6A).

Some nerve/nerve synapses seem very simple (Plate 8.6B) with no modifications of the area of contact apart from parallel membranes and a concentration of inclusions on one (presynaptic) side of the synapse. Others reveal more complex organisations. In almost all nerve/nerve synapses the pre-synaptic region contains small clear vesicles alone or a great majority of them.

Neuromuscular junctions have been found less frequently than

nerve/nerve synapses. They have been seen to occur between inclusion-packed dendrites that are assumed to be the terminations of motor axons near the non-fibrillar sarcoplasmic portion of striated muscle fibres. A few examples of possible neuromuscular junctions have been seen between nerves and non-striated muscles (Plate 8.7). The sarcoplasmic portion of the muscle fibres sometimes extends for a considerable distance from the contractile elements of the muscle fibres before making contact with the nerve. Multiple synapses on a single sarcoplasmic extension have been observed (see, for example, Plate 8.9) and in a number of cases sarcoplasmic processes from more than one muscle fibre, have been seen associated with a single nerve ending (Plate 8.11).

The neuromuscular junctions of the caudal striated muscle cells show a range of possible organelle configurations. At least five different major patterns are identifiable, with respect to:

a) the nature of the post junctional (sarcolemmal) membrane,
b) the organisation of the cleft between pre- and post-junctional membranes,
c) the population structures of the vesicles in the pre-junctional terminal, and
d) the presence or absence of complex pre-junctional structures subtended the area of junctional contact.

The five main appearances are illustrated in Plates 8.8-8.11 and their possible features are summarised in Table 8.1 and Figure 8.2).

The most striking aspect of multiple nerve contacts with a single muscle sarcoplasmic process is the presence of two populations of nerve endings which can be distinguished by their vesicle content. In one the pre-synaptic terminal shows a large number of small clear vesicles only, while the other contains a cluster of medium sized dense-cored inclusions together with few small clear vesicles.

Examples have been observed in which processes from a number of striated muscle cells appear to converge on a single nerve ending packed with dense-cored inclusions and few clear vesicles. In Plate 8.11, for instance, such processes from three separate muscle cells come close to a single nervous terminal. The contact between one muscle process and the terminal appears to be clearly junctional with the muscular post-junctional membrane revealing

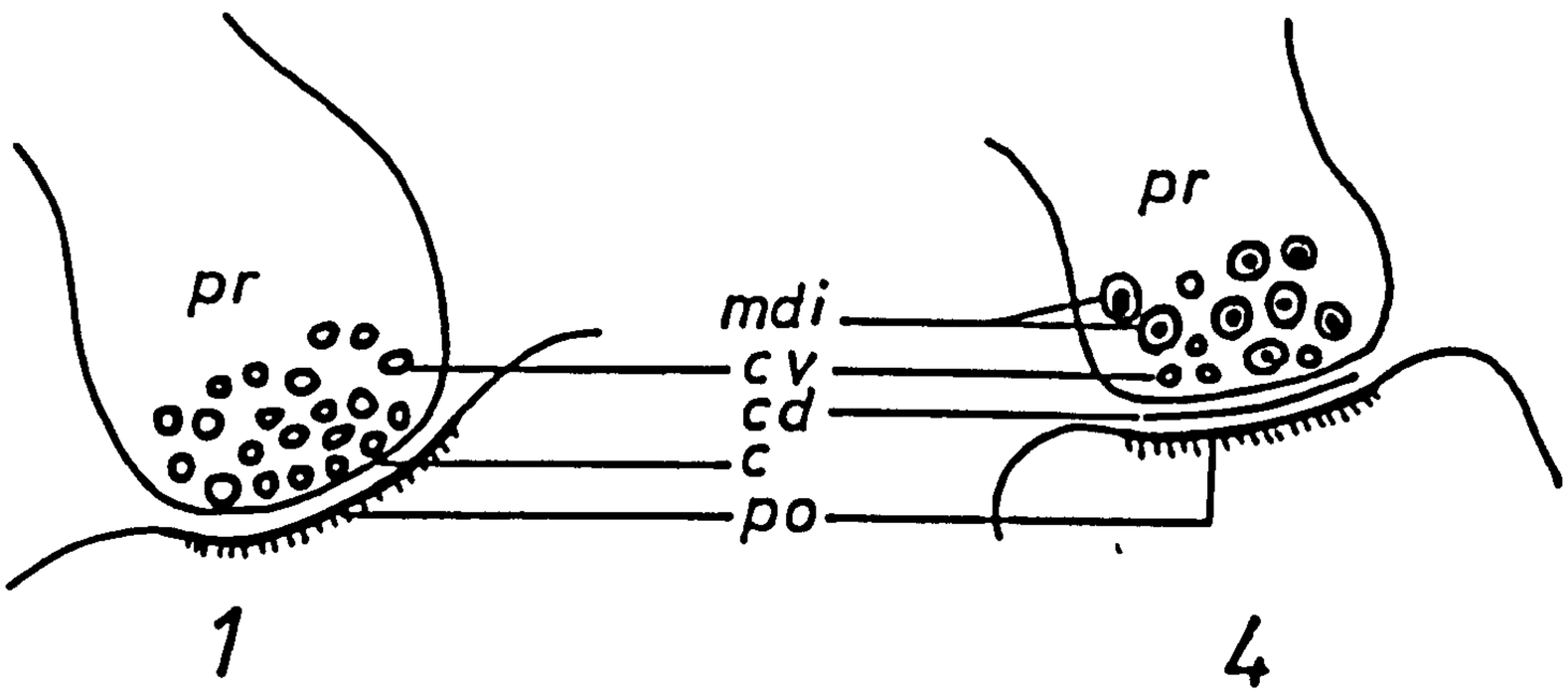
Table 8.1 The attributes of five types of neuromuscular junction observable in the tails of T. patialense cercariae.

Types of neuromuscular junction	Pre-synaptic structure (+)	Pre-synaptic inclusions C-clear vesicle Dc-dense cored vesicle	Synaptic cleft				Post-junctional thickening with tuft-like dense projection (+)
			Cleft width (nm)	Simple cleft (+)	Filled cleft (+)	Central cleft structure (+)	
1	-	C	34	+	-	-	+
2	+	C	no cleft	-	+	-	+
3	-	C	34	-	-	+	+
4	-	C+Dc	15	-	-	+	+
5	-	C+Dc	23	+	-	-	+

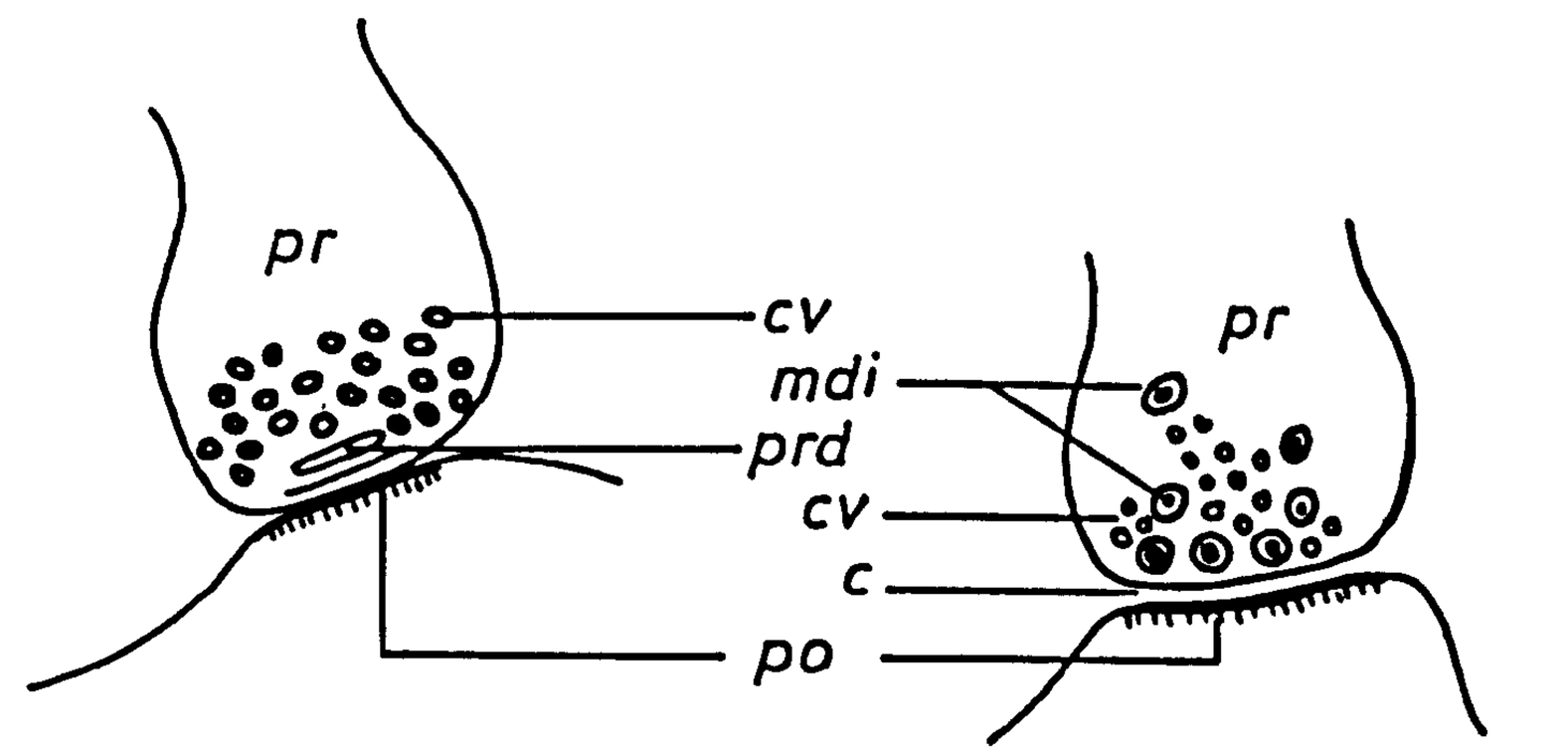
Figure 8.2 Schematic representation of a range of neuromuscular junction types found in T. patialense cercarial tails

1. Type 1 neuromuscular junction
2. Type 2 " "
3. Type 3 " "
4. Type 4 " "
5. Type 5 " "

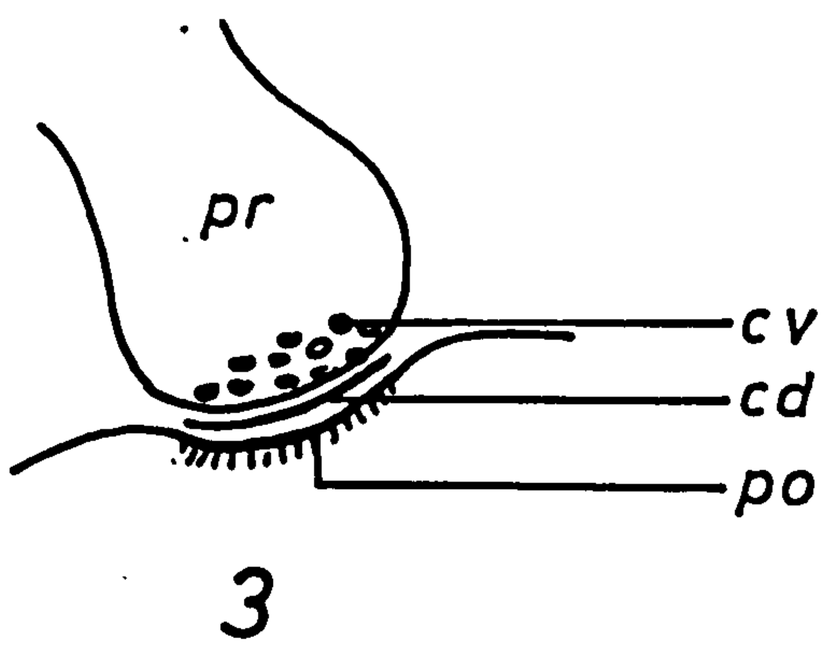
c: cleft; cd: cleft density; cv: small clear vesicle; mdi: medium diameter dense cored inclusion; po: post-synaptic membrane; pr: pre-synaptic terminal; prd: pre-synaptic density.



1 4



2 5



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increased electron density (Type 5 neuromuscular junction). Although the other two processes come into close contact with the terminal in the section available no membrane specialisation are obvious, although they may be present out of section.

The five types of neuromuscular contact associated with caudal striated muscles described above are undoubtedly neuromuscular junctions. On a few occasions apparent contacts have been found between dendrites and regions of caudal, non-striated circular muscle. These contacts may represent true neuromuscular junctions but their simple organisation leaves open the possibility that they are functionally unimportant cell contacts. Plate 8.7 illustrates such an area of close contact. No specialised pre- or post-synaptic structures are apparent.

8.3.3 Sensory structures

8.3.3.1 Ocelli

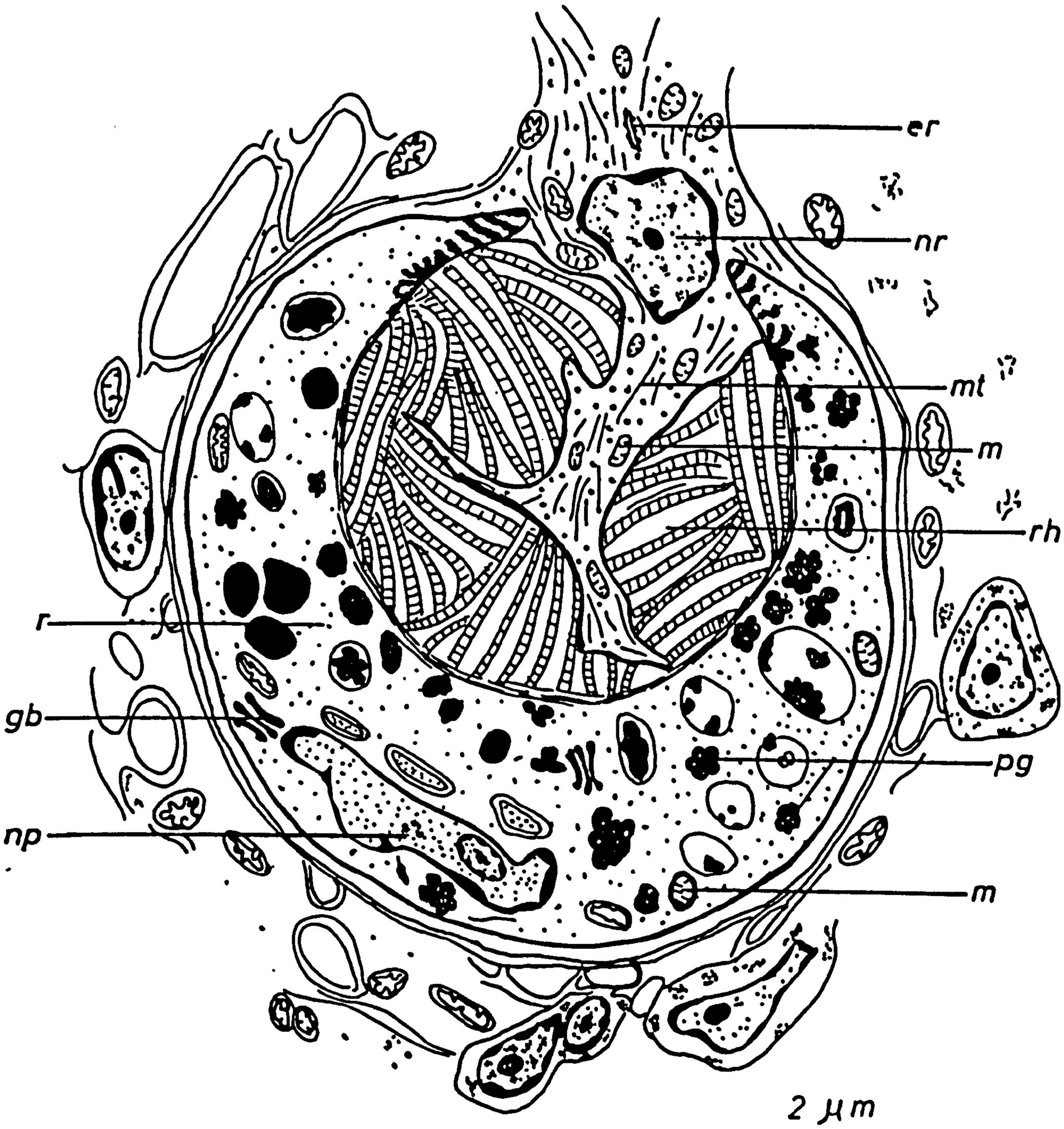
T. patialense cercariae have a pair of ocelli with pigment cups situated near the anterior end of the dorsal surface of the body. Their general structure as revealed by TEM is shown schematically in Figure 8.3. Each ocellus consists of unicellular pigment cup about 21 µm in diameter and apparently one retinular cell. The openings of the pigment cups are directed postero-laterally. The cytoplasm of each pigment cell is occupied largely by round or oval pigment vesicles (Plate 8.12). The inclusions within the vesicle appear as small aggregations of granules which do not completely fill the vesicles. The pigment cell nucleus lies peripherally near the convex outer surface of the cell, in a small region of cytoplasm without pigment vesicles (Plate 8.13). Mitochondria, golgi bodies,ribosomes and microtubules are encountered in the pigment cell cytoplasm.

Near the bottom of the cup, the retinular cell consists of a closely packed array of microvilli, collectively termed the rhabdomere (Plate 8.12). The microvilli appeared to be in groups extending in various directions from the flattened central region. Within each group the microvilli are approximately parallel and have clup-shaped distal ends (Plate 8.14A).

The cytoplasm of the central portion of the retinular cell contains mitochondria, microtubules and granular endoplasmic reticulum. External to the rhabdomeric chamber within the pigment cup, the retinular cell enlarges, its cytoplasm has an alveolar

Figure 8.3 Diagrammatic representation of an ocellus of a T. patialense cercaria (based on transmission electron micrographs).

er: granular endoplasmic reticulum; gb: golgi body; m:mitochondrion; mt: microtubules; np: nucleus of pigment cup cell; nr: nucleus of retinular cell; pg: pigment granules; r: ribosomes; rh: rhabdomere


er
nr
mt
m
rh
r
gb
np
pg
m
2 µm

appearance and contain an increased number of mitochondria, microtubules as well as the nucleus which is about 5.5 μm in diameter and is situated in the mouth of the cup (Plate 8.12 and 8.13).

Dense structures thought to be desmosomes occur between retinular and pigment cells at the openings of the rhabdomere chamber (Plate 8.14B). Near the opening of the pigment cup, a large asymmetrical accumulation of electron-dense material is apparent in the pigment cup cell cytoplasm and close to such presumed desmosomes. Profiles through this material show that it consists of alternating electron-dense and electron-lucent striations which presumably provide some extra strength to the junctional region.

8.3.3.2 Surface receptors

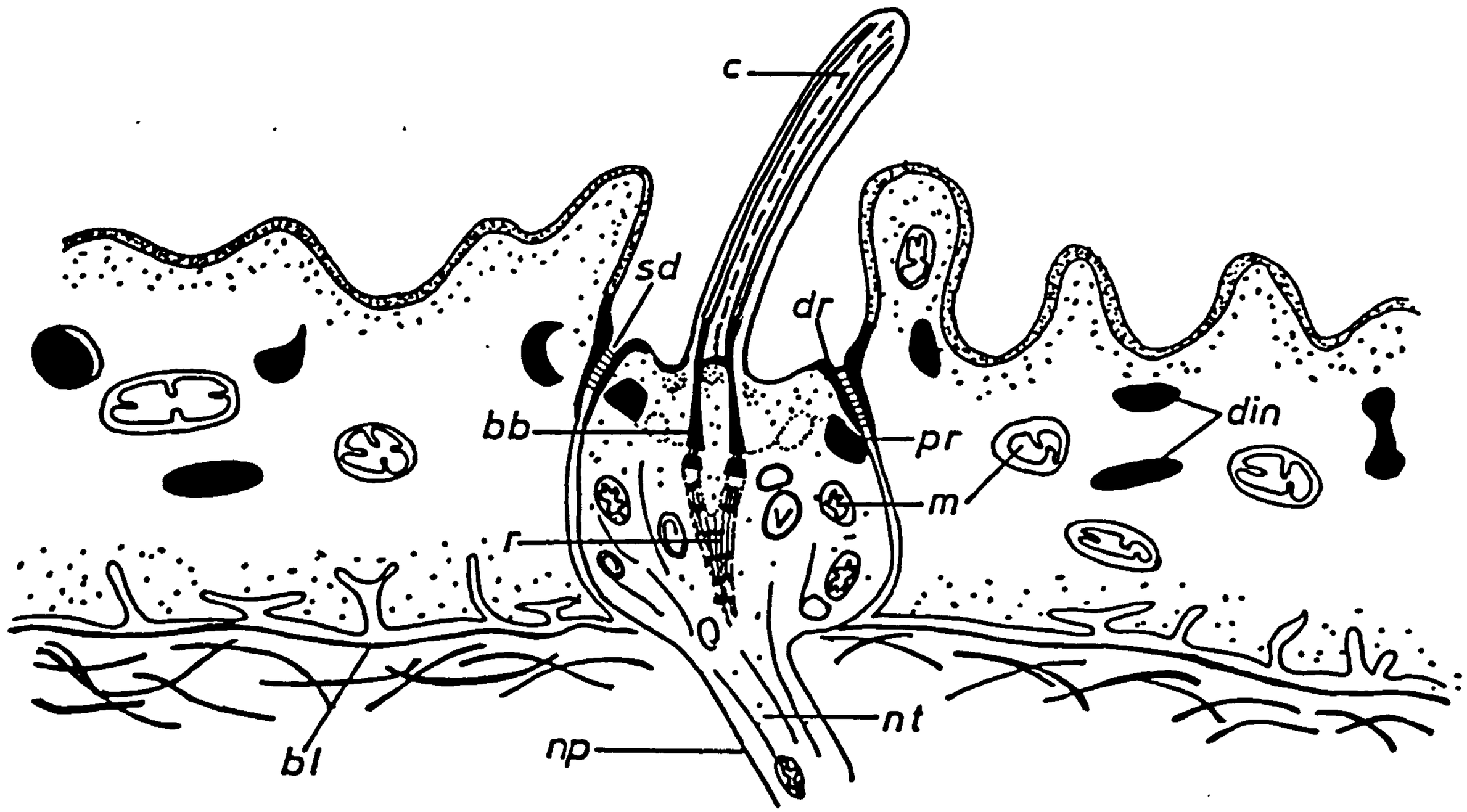
Uniciliated sensory endings were found distributed in the tegument of the body, tail stem, arm processes and furcae of the tail. In all cases their cilia protruded through apertures in the distal cytoplasm of the tegument of the cercariae.

Each uniciliated sensory ending consists of a sensory bulb of cytoplasm with a single projecting cilium (Figure 8.4, Plate 8.15A). In some sections the base of the bulb is seen to penetrate the basal lamina as a neural process with many parallel neurotubules (Plate 8.15B). Bulbs are approximately 1.3 μm in diameter and enclosed laterally by the surface tegument, to which they are attached by a ring of septate desmosomal contact. Associated with this contact area is a ring of electron dense material on the inner side of the membrane of the bulb. The plasma membrane of the tegument is thickened adjacent to the outer side of the junction. An additional electron dense ring is usually present in the bulb just below the first ring and may be fused with it. As well as these dense annuli, the bulb cytoplasm also contains mitochondria and small electron-lucid vesicles about 110 nm in diameter.

In sections, the terminal cilium had a diameter of about 0.22 μm and extended 1.4-4.5 μm from the bulb, although it is likely (see Whitfield et al., 1975) that they can extend much further than this. The sensory cilia appear to have a 9+2 microtubular arrangement. At the base of the cilium there exists

Figure 8.4 Schematic diagram of uniciliated nerve ending in the surface tegument of the tail stem of a cercaria. (Based on transmission electron micrographs.)

bb: basal body; bl: basal lamina; c: cilium; din: electron dense inclusion; dr: distal ring; m: mitochondria; np: nerve process; pr: proximal ring; r: rootlet; sd: septate desmosomes.



0.5 µm

both a basal plate and a dense basal body. The basal body appears in longitudinal section as two columns of electron dense material running from a position at the base of the cilium process to the point of insertion of the striated rootlet (Plate 8.15A). In cross section the basal body appears as a hollow tubular electron-dense structure, lying centrally within the chamber of the nerve bulb.

8.4 Discussion

8.4.1 The ultrastructure of the nervous system of T. patialense cercariae.

The present study showed that well developed central and peripheral nervous systems are present in T. patialense cercariae with demonstrable contacts with muscle cells and presumed connections with a variety of sensory endings. These linked organ systems must be assumed to provide the cercariae with a mean for receiving information from the external (and possibly the internal) environment integrating these sensory inputs and coordinating effector organ behavioural responses.

The central nervous system is seen to consist of a peripheral layer of cell bodies enclosing a central fibrous core. This type of distribution was also noted in other cercariae by Rees (1967), investigating Parorchis acanthus and Nuttman (1975) in studies on Schistosoma mansoni. A similar type of organisation has been described in the cerebral ganglion of Fasciola hepatica miracidia (Wilson, 1970).

The cerebral ganglia presumably act as an integrating centre for the various afferent sensory inputs. It can also be assumed that parts of this integrative activity involve the initiation of appropriate components of spontaneous behaviour as well as behaviour elicited by particular sensory inputs. The nerve masses at the proximal and distal ends of the tail stem might have similar functions, but ones restricted to the activities of the tail.

8.4.1.1 The ultrastructure of individual neurons

The intracellular contents of the nerve axons and dendrites resemble those described for some other free-living and parasitic invertebrates e.g. annelids (Hagadorn, 1962), arthropods (Smith & Treherne, 1963), molluscs (Gerschenfeld, 1963), Fasciola hepatica cercariae and miracidia (Dixon & Mercer, 1965 and Wilson,1970, respectively),

Schistosoma mansoni cercariae (Nuttman, 1975), Cercaria linearis and Cercaria stunkardi (James, 1975), Cryptocotyle lingua (see Rees, 1975). The small clear vesicles are similar in size and appearance to the cholinergic synaptic vesicles found in vertebrate nerves (Whittaker & Gray, 1962; De Robertes, 1964; Robertson, 1964), molluscs (Gerschenfeld, 1963) and other larval digeneans (Dixon & Mercer, 1965; Wilson, 1970; James, 1975; Nuttman, 1975; Rees, 1975). None of the cell body types (1A, 1B or 2) associated with the cerebral ganglia or longitudinal nerve cords contain vesicles of this type. This presumably means that their synthesis occurs distal to the cell body, in the nervous processes or within the cell body cytoplasm in a manner that does not reach the stage of identifiable vesicles.

In view of the close morphological similarity between the small clear vesicles seen in this study and proven acetylcholine-containing vesicles from previous studies or, in particular, vertebrate systems, it is assumed here that this category of nerve cell inclusion does contain this transmitter. Supporting this assumption is the fact that acetylcholinesterase activity, as deduced by the relatively specific acetylthiocholine iodide technique, is found in all the regions of the nervous system where small clear vesicles are apparent.

The medium size dense-cored inclusions are close in appearance to inclusions present in numerous invertebrates nerve fibres, e.g. molluscs (Gerschenfeld, 1963; McKenna & Rosenbluth, 1973), turbellaria (Reuter, Wikgren & Palmberg, 1980; Reuter, 1981) and other cercariae (Dixon & Mercer, 1965; James, 1975; Nuttman, 1975). The dense-cored inclusions are interpreted as containing catecholamines. Catecholamines have been demonstrated in most parts of the nervous system of T. patialense cercariae by fluorescence histochemical methods (see Chapter 5). Such methods when applied to whole mounts of cercariae suggest a distribution of catecholamines which correspond well with the ultrastructural distribution of neurons containing dense-cored inclusions. The latter are generally regarded as the storage sites of biogenic amines like catecholamines. The catecholamines which commonly serve as neurotransmitters in vertebrates central nervous systems are most likely primary amines (dopamine, noradrenaline) since the green fluorescence can be induced by a short time (one hour) exposure to formaldehyde vapour and no increase in the intensity of fluorescence after three hours

exposure to indicate secondary amines (adrenaline)(Falck & Ohman, 1965).

The kinetics of the production of formaldehyde-induced fluorescence in T. patialense cercarial nervous systems is similar to that of the vertebrate central nervous system. It is thus concluded that the catecholamines present are more likely to be dopamine and noradrenaline rather than adrenaline.

Indeed, dopamine has been shown to be the dominant catecholamine in turbellarians, while noradrenaline seems to be present at lower concentration (Welsh & Kings, 1970). In addition, biogenic amines have been reported in sensory neurons of turbellaria. (Welsh & Williams, 1970) and according to Kerkut (1973), dopamine is by far the most important catecholamine in invertebrate nervous systems and probably acts as a neurotransmitter. There is no clear evidence indicating the occurrance of adrenaline in any invertebrate neurons (Gersch, 1975). Apart from their function as conventional neuro-transmitters, biogenic amines are believed to play a role in the release mechanism of peptide neurosecretion (Gersch, 1975). All the above evidence pointing to dopamine being the most likely candidate as a T. patialense catecholamine transmitter, has, however, to be set against the findings of Chapter 6 which showed that adrenaline was the most inhibitory catecholamine in direct pharmacological tests.

Type 1B cells of the cerebral ganglia and longitudinal nerve cords contain large numbers of medium diameter dense-cored inclusions. It is difficult to avoid the conclusion that these cells are synthesizing such inclusions and that they contain catecholamines.

The large dense inclusions bear a striking resemblance to the neurosecretory vesicles described from many animal groups, e.g. annelids (Hagadorn, 1962), molluscs (Gerschefeld, 1963; McKenna & Rosenbluth, 1973), larval digenea (Dixon & Mercer, 1965; Wilson, 1970), turbellaria (Morita & Best, 1965; Reuter, 1981), cestodes (Marseth, 1967; Webb, 1976, 1977; Fairweather & Threadgold, 1981), crustacea (Knowles, 1962). In fact, all of the well substantiated neurosecretory systems which have been described in animals so far have been characterised electron microscopically by such inclusions. It is likely the large dense inclusions described here also could be neurosecretory. Although there is no direct physiological evidence for such a function, it is interesting to speculate about

cercarial processes which could be under neurosecretory control. It is possible, for instance, that the processes of growth and precocious sexual maturation in the cercariae of T. patialense might be partly controlled by neurosecretory activity. Lender (1974) has demonstrated that in turbellarians neurosecretions seem to be involved in the control of asexual multiplication, growth and sexual maturation.

On the basis of histochemical tests involving the application of aldehyde-fuchsin or chrome-haematoxylin-phloxin technique, neurosecretory cells have been demonstrated in digeneans (see, for example, Ude, 1962; Matskasi, 1970; Harris & Cheng, 1972; Shyamasundary & Hanumantha Rao, 1975; Sharma & Sharma, 1981) and in cestodes (Davey & Breckenridge, 1967; Webb, 1977; Fairweather & Threadgold, 1981). In the present study, however, using both of these staining techniques it has not been possible to identify cells reacting positively to these stains.

The type 2 cells of the cerebral ganglia and longitudinal nerve cords sometimes contain large diameter dense inclusions as well as extensive granular endoplasmic reticulum with fine granular contents. This association of organelles suggests that the type 2 cells are the cell bodies connected to the nerve processes containing large diameter dense inclusions, which as stated above, are likely to be neurosecretory in nature. It is likely that the protein contents of the presumptive neurosecretory inclusions are being synthesized in the perinuclear cytoplasm of the type 2 cells.

The release sites of neurosecretory material have been identified by many workers to be accomplished by exocytosis (Norman, 1976; Webb, 1976, 1977; Fairweather & Threadgold, 1981; Reuter, 1981). No such areas of neurosecretory material release have been identified in this study.

8.4.1.2 Synapses and neuromuscular junctions

Typical synaptic structures between adjacent nervous processes are found in the main ganglia, nerve masses and nerve cords of T. patialense cercariae. They are identified by their characteristic accumulation of vesicles in the presynaptic fibre and the dense thickening of the post synaptic membranes. These distinctive synapses are similar to those of many other animal species. The synapses without thickenings are more difficult to classify. However, reports of synapses without

post-synaptic thickenings in turbellarians (Moraczewski, Czubaj & Bakowska, 1977) support the interpretation of this structure as a form of true synapse. On the basis of the type of inclusion associated with most of the nerve/nerve synapses (i.e. small, clear vesicles), it is assumed that most such synapses are of the cholinergic type.

Previous studies of neuromuscular junctions (e.g. Robertson, 1956; Birks, Huxley & Katz, 1960; Richardson, 1962; Graziadei, 1966; Uchizona, 1967; Padykula & Ganthier, 1970) have demonstrated two morphological features which are common to junctions in a variety of muscle types and a variety of animals. These constant components are: proximity of the nerve and muscle plasma membranes with no cellular processes intervening and the presence of large numbers of vesicles in the junctional region of the axon. Variable features include the exact dimensions of the gap, the presence of formed extracellular material within the gap, specialisation of the junctional membranes, and variations in the morphology of the vesicles.

In T. patialense cercariae a peculiar kind of neuromuscular junction has been identified which has previously only been recorded from a small number of unrelated animal groups such as Ascaris (Debell, 1965; Rosenbluth, 1965b), Amphioxus (Flood, 1966), echinoderms (Cobb & Laverack, 1967), earthworms (Mills & Knapp, 1970b) and the turbellarian, Dugesia tigrina and Notoplana acticola (MacRae, 1963 and Chien & Koopowitz, 1972 respectively. In all these animals some or all of the neuromuscular junctions are formed between motor nerves and sarcoplasmic extensions of the muscle cells. To a variable extent, the muscle cell is responsible for spatial extension towards to a junction area, in these instances. Chien & Koopowitz (1972) have proposed the term sarconeural junction for the juxtaposition between sarcoplasmic extensions and nerve terminals to distinguish them from the more usual neuromuscular junctions.

In the present study, neuromuscular junctions are characterised by a junctional gap of 15-34 nm containing no collagen fibrils or basement membrane. Other junctional specialisation, however, including mid-cleft material and specialisations of the pre- and post-junctional membranes occur in some examples.

The most important differences between the neuromuscular synapses of T. patialense cercariae and those of vertebrate striated muscle is the absence in the former of the subsynaptic sarcolemmal infoldings. A feature common in other invertebrate muscles such as the femoral muscles of Vespula carolina (Edwards, Ruska & de Harven, 1957) and vertebrate smooth muscle (Caesar, Edwards & Ruska, 1957).

The sarcoplasmic processes receive synaptic endings from one or more dendrites and presumably conduct excitation toward the contractile parts of the cells. With multiple innervations possible, it may be suggested that the extensions potentially involved with integrative processes.

One important finding of the present study linked with structure and function of neuromuscular junction was the finding of a doubly innervated sarcoplasmic extension. Two neuromuscular contact regions, spaced about 4.5 μm apart were present on a single extension (see Plate 8.9). One prejunctional terminal contained small clear vesicles, the other mainly dense-cored inclusions. It is tempting to suggest that this organisation represents a pair of innervations with antagonistic functions. One, for instance, might be excitatory, the other inhibitory with the sarcoplasmic extension representing a zone of integration of nervous inputs. It is interesting to note, in this context, that both neuromuscular inhibitory as well as excitatory events have been described in polyclads (Koopowitz & Ewer, 1970).

Another interesting finding reported in this chapter relates to the multiple innervation of striated muscle cells by a single nerve terminal. In one instance, (see Plate 8.11) an organisation was seen that suggested that three different striated muscle cells shared a single junctional neurone terminal containing medium diameter dense-cored inclusions. This pattern of neuromuscular contact would imply that such muscles were able to be integrated into functional groups by shared neuromuscular innervations. Such integration could also be achieved within the central nervous system with separately innervated muscle cells, but one other integration method seems possible on a local basis. In Chapter 7, it was shown that adjacent striated muscle cells sometimes show regions of close sarcolemmal contact (an intermembrane gap of about 25 nm). Such close contacts raise the possibilities

that action potentials in one muscle cell could elicit a similar depolarization of adjacent cells by electrical coupling. If such a mechanism was indeed present, it would be possible for some of the muscle cells to remain uninnervated yet still be functionally integrated with other, innervated cells. Such a system would be equivalent to that occuring in some smooth muscle cells of vertebrates (McKenna & Rosenbluth, 1973).

It thus appears that at least three distinct modes of functional integration of muscle cell contraction are present in T. patialense tail striated muscle cells. These three modes are illustrated diagrammatically in Figure 8.5.

Previous efforts to distinguish different functional types of neuromuscular junctions morphologically have been disappointing. Peterson & Pepe, 1961, for example, examined inhibitory nerve endings in crayfish and found them not to be significantly different in appearance from excitatory terminals. In T. patialense cercariae neuromuscular junctions two populations of nerve endings can be distinguished by their vesicles content but consistent differences in gap width and post-junctional membrane specialisation are not present.

Richardson, 1962; 1964 showed that the smooth muscle of the rat vasdiferens, which is known to receive an adrenergic nerve supply, possesses neuromuscular junctions marked by large numbers of dense-cored vesicles, whereas in the iris, which receives an almost purely cholinergic innervation, the nerve endings contain clear vesicles primarily. Other details of these junctions, i.e. gap width and post-junctional specialisation, are, however, not noticeably different. In the somatic muscles of Ascaris and Lumbricus, which are known to have a cholinergic innervation, endings with large numbers of clear vesicles can also be found (Rosenbluth, 1965; 1972).

It is most probably that in T. patialense cercariae, junctions with nerve endings containing clear vesicles are sites of cholinergic transmission while junctions with axon terminals containing an abundance of dense-cored inclusions are probably sites of catecholaminergic transmission.

The mixture of clear and dense-cored vesicles seen in the nerve endings of cercariae is not an unusual phenomenon. Clear vesicles are found in mammalian peripheral noradrenergic terminals

Figure 8.5 Diagrams illustrating possible modes of functional integration of striated muscle cells in T. patialense cercariae.

a) Integration of CNS level

CNS neurons "driving" a functionally related group of motor axons

b) Integration at neuromuscular junction level

A single motor neuron terminal makes contact with sarcoplasmic extensions from several functionally related muscle cells

c) Integration at the muscle cell level

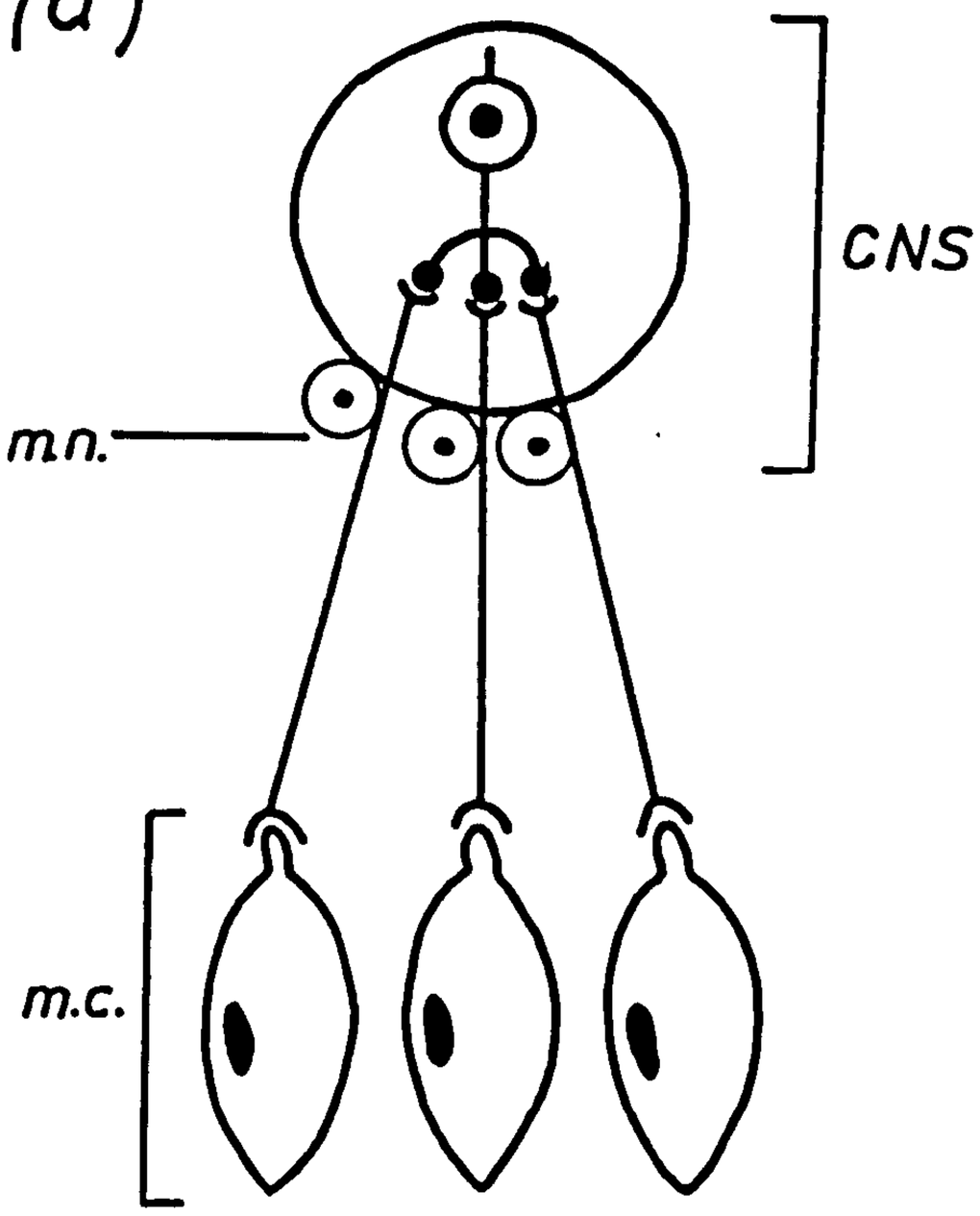
Activation of one muscle cell by a neuromuscular junction stimulates adjacent cells by electrical couplings between muscle cells

CNS: central nervous system

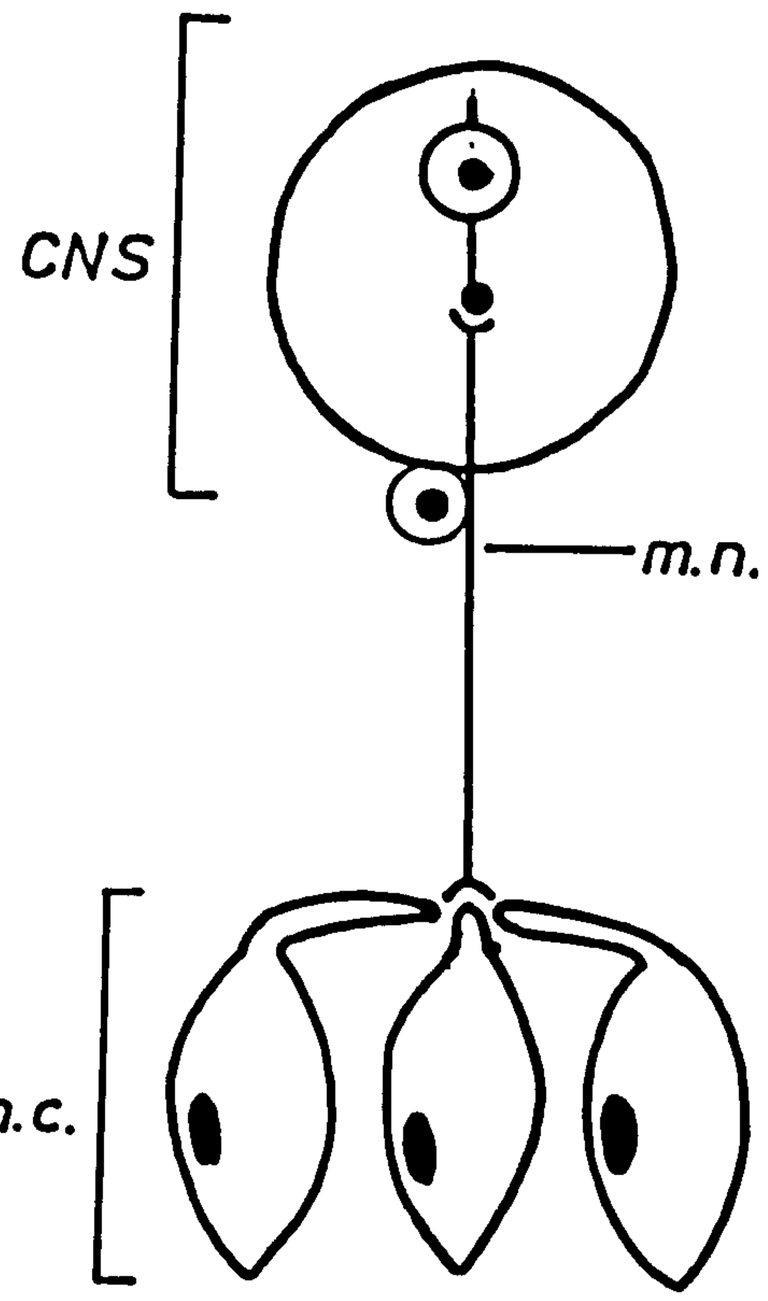
mc: muscle cells

mn: motor neurons

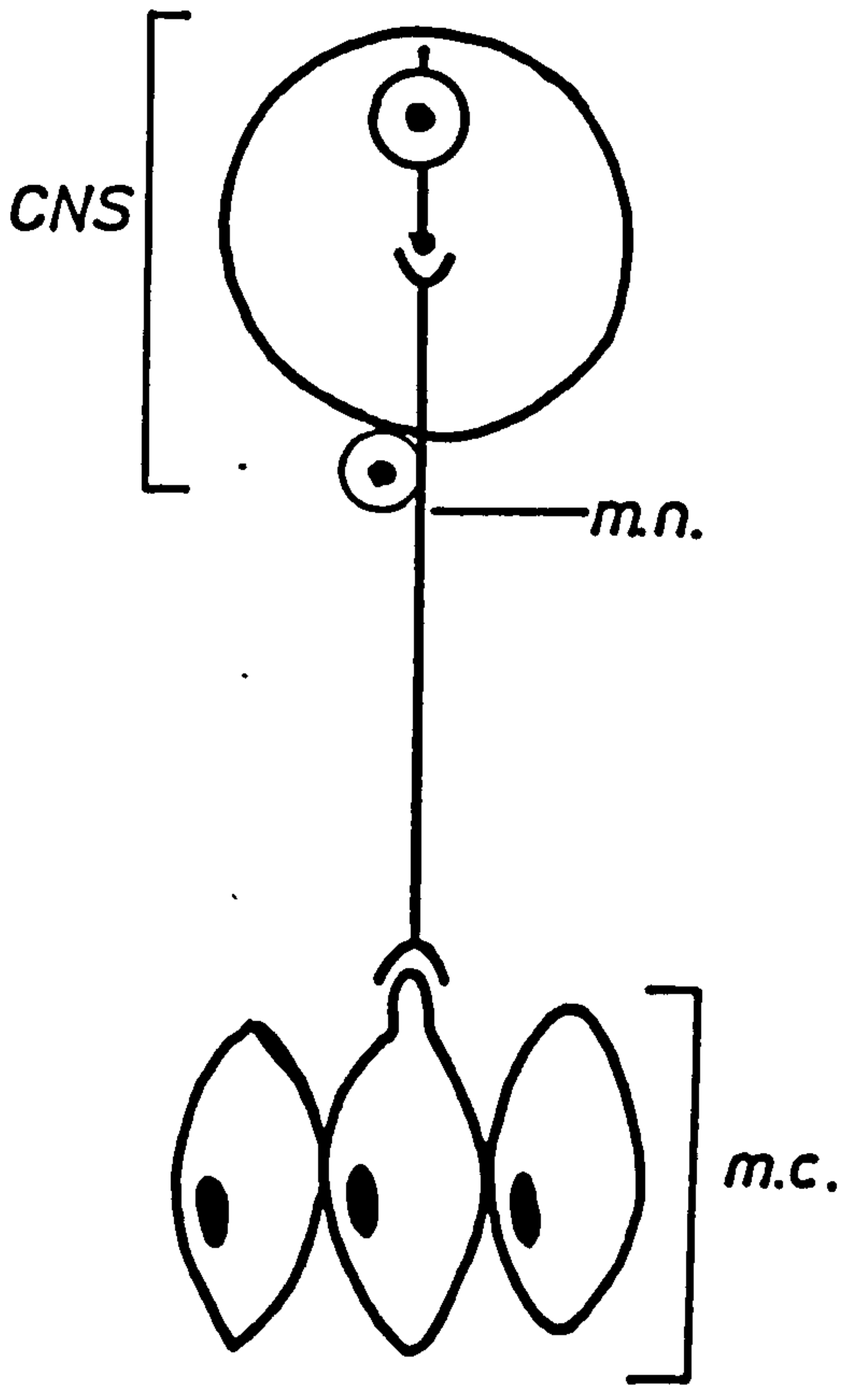
(a)



(b)



(c)



as well as in neurosecretory endings (Richardson, 1964) while an occasional dense-cored vesicle is found in cholinergic endings in vertebrate skeletal muscles (Birks, Huxley & Katz , 1960).

The occurrence of both vesicle types in different endings raises a question about their function in each case. The clear vesicles found in cholinergic endings presumably represent packets of acetycholine while in presumptive catecholaminergic terminals they may represent membrane recaptured after neurotransmitter discharge or immature catecholamines storage organelles which will develop a dense core and increase in size after its catecholamine accumulation. Conversely, the dense-cored vesicles present in catecholaminergic neurons are presumably the storage organelles for catecholamines, whereas in cholinergic nerve endings they may be related to the metabolism of the ending or contain trophic substance (McKenna & Rosenbluth, 1973).

8.4.1.3 Sensory structures

(a) Ocelli

Photoreceptors in the animal kingdom appear to have been developed along two different evolutionary lines, ciliary and rhabdomeric. Ciliary photoreceptors develop from neuralcilia while the rhabdomeric photoreceptors are produced by specialisation of neural microvilli. (Eakin, 1963). Both types of photoreceptor organ have been described on a number of occasions from larval stages of both digenean and monogeneans.

Pigmented rhabdomeric eyes, that is eye with pigment cups, are a conspicuous feature of the larvae of both these parasitic groups. Investigation on the fine structure of rhabdomeric photoreceptors have been recorded on the miracidia of Fasciola hepatica (see Kummel, 1960; Isseroff & Cable, 1968), Philophthalmus megalurus (see Isseroff, 1963, 1964a), Heronimus chelydrae (see Isseroff, 1964b, 1968), Allocreadium lobatum and spirarchis sp (see Isseroff, 1968) and Diplostomum spathaceum (see Brooker, 1972). Similar studies have been carried out on the cercariae of Macrovestibulum eversum; Skrjabinopsolus manteri and Crepidostomum sp (see Pond & Cable, 1966) and Spirorchis sp (see Isseroff & Cable, 1968), and on the oncomiracidium of Entobdella soleae (Kearn & Baker, 1973). In the larvae of these parasitic platyhelminths the eyes are made up of one to five rhabdomeres shielded by cells containing pigment

granules. It appears that diversity of photoreceptors orientations in cercariae, for instance, may be correlated with the varied patterns of photostatic behaviour shown by these larvae. In T. patialense cercariae, each ocellus appeared to consist of a single rhabdomere surrounded by a single cup-shaped pigment cell. This structure resembles that of the eye spots of Crepidostomum sp cercariae (Pond & Cable, 1966).

The junctional complexes resembling desmosomes serve to bind the retinular cell of T. patialense cercarial eyes to the pigment cell at the point where the retinular cell emerges from the pigment cup. Similar junctions have been described in corresponding locations in the pigmented eyes of several other digenean larvae (Isseroff, 1964a; Isseroff & Cable, 1968). Lowenstein & Kannyo, 1964, and Lasansky, 1967, have suggested that these cellular contacts between retinular cells and pigment cup cells could provide a site for electrical coupling between them. They did not, however, speculate about the functional significance of such coupling. The primary function of the pigment cup must be to regulate the amount and orientation of the light impringing upon the photosensitive rhabdomere. If these two aspects of the illumination are to be adaptively altered by the pigment cup cells they require information about light intensity. The cells could respond directly to light but it is perhaps more likely that they obtain such information indirectly from the rhabdomeric cells. If this is so, the desmosomal contacts with the postulated electrical coupling that they allow, could possibly be the means of such information transfer. Carpenter, Moritd & Best (1974) have shown that some transfer of this type might be going on in the eyes of planarians. More specifically, one might hypothesize that in low ambient light levels pigment granules could move along from the desmosomal zone, thus increasing the aperture of the ocellus and its sensitivity. In high light intensity, in contrast, granules could move towards the desmosomal region protecting the rhabdomere from excessive illumination and increasing orientation specificity.

In cercariae other than those of T. patialense eyes, when present, are often of a complex retinular organisation with more than one rhabdomeric cell and with these showing differing orientations.

The T. patialense cercarial eye is much simpler in structure with a single rhabdomeric cell and cup orientation for each eye. This orientation of the pigment cup is postero-lateral in the transverostrematid and has to be compared with the normal, principally antero-lateral orientation of the rhabdomes of other cercariae. It is interesting that this inversion of orientation pattern coincides with the flexed body conformation of T. patialense cercariae. The flexure means that in tail first swimming both normal ocellate cercariae and those of T. patialense swim with their eyes, major acceptance cones point postero-laterally with respect to the direction of swimming. This relationship is thus concerned in the transversotrematid condition although its functional significance is unknown.

Anderson & Whitfield (1975) found that T. patialense cercariae swimming activity can be initiated by sudden changes in light intensity such as the movement of a shadow over the ocelli. So it is possible that the pigmented rhabdomeric eyes could provide crude information on the direction of incident light and respond to reduction in illumination.

Among digeneans the unusual feature of transversotrematid pigment cup ocelli is the apparently unique persistence of these eye spots into and through the adult stage. All species of the genus Transversotrema are now known to possess this characteristic. The original description of the marine sp. T. haasi by Witenberg (1944) failed to record the presence of ocelli in the adult, but a recent description by Velasquez (1975) confirmed that this species is also biocellate. It is likely that this most unusual retention of eyes into digenean adulthood among the transversotrematids is linked to their equally unusual, essentially ectoparasitic location upon fish. In their sub-scale recesses, they are certainly exposed to light from the outside world. Indeed, (Whitfield, per.comm) adult T. patialense under the translucent scales of Brachydanio rerio will move away from a cold light source focused on their location, so it appears that light is certainly perceived by these adult flukes. As it is vital for the adults to remain under their hosts scales, as negative phototaxis linked to the receptor abilities of the eyes would adaptively act to keep the adults in this position.

It has been shown (Chapter 4) that light to dark changes

play an important role as cues for an endogenous circadian emergence rhythm in the cercariae of T. patialense. It is possible that the pigmented receptors of the cercariae within the tissue of the snail may act as recorders of transitions between day and night and be involved in the control of the circadian activity.

(b) Surface receptors

Ultrastructural examination of the tegument of T. patialense cercariae during this study has revealed presumed sensory receptors extending through the distal cytoplasm of the tegument of both the head and tail. Apart from the complex, macrocilium-containing mamilliform receptors of the arm process tips (see, Whitfield etal., 1975) all of these presumed receptors consist of cytoplasmic terminal bulbs, each with a single cilium. Similar structures, always presumed to represent sensory endings have been described at the ultrastructural level from cestodes (Morseth, 1967; Blitz & Smyth, 1973; Cooper, Allison & Ubelaker, 1975), monogeneans (Holton & Morris, 1969; Lyons, 1969a and b), adult digeneans (Erasmus, 1967, 1969, 1970; Morris & Threadgold, 1967; Smith, Reynolds & Lichtenberg, 1969; Silk & Spencer, 1969), miracidia (Wilson, 1970; Brooker, 1972; Bundy, 1979) daughter sporocysts (Imohiosen, 1969; Matricon-Gondron, 1971; James, 1975), rediae (Imohiosen, 1969; James, 1975) and cercariae (Dixon & Mercer, 1965; Morris & Threadgold, 1967; Chapman & Wilson, 1970; Køie, 1971; Matricon-Gondron, 1971; Morris, 1971; Nuttman, 1971; Bebby & Rees, 1975; Whitfield, Anderson & Moloney, 1975; Bundy, 1979). The normally multiciliate or clustered endings usually referred to as sensory pits in other parasitic platyhelminths (see Nuttman, 1971) have not been seen in this study.

Attempts to demonstrate that presumed uniciliate receptors have differing detailed structure in the different taxa by considering such aspects of this ultrastructure as ciliary rootlets and the number and size of membrane bound vesicles have met with limited success (Matricon-Gondron, 1971). Neither of these characters is satisfactory since sectioning artifacts make precise interpretation difficult (Weibel, 1969).

At the light microscope level many workers have used silver nitrate staining technique to locate and visualise presumed sensory endings in a variety of parasitic platyhelminths (Wagner, 1961; Lie, 1966; Rohde, 1968; Chapman & Wilson, 1970; Short & Cartlett, 1973;

Whitfield, 1979; Grabda-Kazubska & Moczon, 1981; Niewiadoniska & Moczon, 1982). The sensory papillae visible under the light microscope are found to be corresponding in position to sensory structures in the tegument described by scanning electron microscope (Wagner, 1961; Lie, 1966; Chapman & Wilson, 1970).

The ciliated endings at the surface of parasitic platyhelminths have been presumed to be sensory receptors on the basis of comparisons with similar structures known on good neurophysiological evidence to function as sensory receptor in other groups. In the absence of any detailed neurophysiological information concerning cercarial ciliated endings, it is impossible to either confirm categorically that they are sensory in function or to come to rigorous conclusions about their modalities. There seems little doubt, though, that they are indeed sensory structures and some speculation about sensory modalities can stem from a consideration of their detailed ultrastructure organisation.

Protruding uniciliate endings of parasitic platyhelminths are generally assumed to function as tangoreceptors (Dixon & Mercer, 1965; Erasmus, 1967; Imohiosen, 1969; Lyons, 1969; Wilson, 1970; Matricon-Gondron, 1971) or rheoreceptors (Morris & Threadgold, 1967; Bebby & Rees, 1973).

The more complex sensory pits are usually regarded as chemoreceptors (Lyon, 1969; Wilson, 1970; Nuttman, 1971).

A previous study on T. patialense cercariae (Whitfield et.al., 1975) suggested that the cluster of mammiliform receptors on the tips of the arm processes are contact chemoreceptors involved in the recognition of fish host surfaces. Anderson & Whitfield (1975) found that T. patialense cercariae in a resting phase become active when the water surrounding them is disturbed. It seems that sensory receptors on the tail or head may be rheoreceptors used for detection of currents in the water. Stimulation is assumed to result in renewed activity of the tail and consequently could increase the chance of contact with a passing fish.

Most of the receptors described in this study are located on the furcae or stem of the tail. Such receptors can be seen in phase contrast preparations of living larvae to possess elongate cilia extending several micrometers from the tegumentary surface. Such an organisation would seem to be ideally constructed to operate

as a rheoreceptor in the fashion described above, i.e. when the cercaria is not swimming. It is difficult to imagine how such receptors could signal useful information about currents when the cercariae itself is actively swimming.

The two rings of nerve endings situated on the attachment surface of the ventral sucker (Bundy, 1979) are presumably to provide information concerning sucker contact with the fish host, while the other sense receptors on the head may be tangoreceptors or chemoreceptors concerned with detection on the appropriate surface for attachment and feeding.

CHAPTER 9

General Discussion

General Discussion

The cercarial/adult generation of transversotrematid life cycles has attracted a considerable amount of experimental attention due largely to the practical advantages of the transversotrematid-melanid gastropod-fish system. Anderson & Whitfield (1975) have enumerated some of these advantages in relation to behavioural and population dynamical experimentation. The advantages include the fact that forms like T. patialense will utilise inexpensive, small and hardy intermediate and definitive hosts and will achieve self-sustaining life cycle maintenance in laboratory aquarium conditions. As well as these benefits related to host and parasite husbandry, the ectoparasitic site of the adult worms provides many experimental possibilities for non-destructive sampling of parasite populations on definitive hosts. Finally, the large cercariae of transversotrematids lend themselves particularly well to handling as individual organisms, so that the examination of the behaviour of single larvae is possible, in some circumstances without the need for microscopy.

Partly as a consequence of this fortuitous collection of features, several aspects of the biology of the cercariae of T. patialense, the subject material of this thesis, have already been described in quantitative detail (see, for instance, Anderson & Whitfield, 1975; Whitfield, Anderson & Moloney, 1975; Whitfield, Anderson & Bundy, 1977; Whitfield, Anderson & Mills, 1977; Bundy, 1981a). In respect of the behavioural dimension of this biology, however, before the work carried out in the present research programme, little was known, if anything, about the activity of cercariae within their snail hosts and during the process of emergence from them. With the results presented in Chapter 3 and 4, it is now possible to say that the total changing sequence of behavioural phases of these larvae has now been described. The sequence starts at the time that they mature within the digestive gland haemocoele spaces of M. tuberculata. It is concluded when the cercariae die or transform into young adults on the surface of a fish.

This thesis has concentrated upon the early stages of the behavioural sequence, a phase in the life span of cercariae about which little is known in any digenean species. In fact the description

in Chapter 3 of the time scale and routes of cercarial movement from digestive gland to rectal sinuses appears to be the only detailed behavioural description of this type of migration for any digenean. Those previous studies which have considered this part of the life cycle in other species have usually concentrated on the sites of emergence. They have not considered the routes by which the larvae arrived there (Kendall & McCullough, 1951; Duke, 1952). Only Probert & Erasmus (1965) working on Cercaria X in Lymnaea stagnalis have provided some information on the intramolluscan migration route. They were unable, however, to give any data on the rapidity or control of this movement.

On the question of emergence itself, understanding of this process in T. patialense cercariae is particularly problematical because of the bodily organisation of these larvae. These highly specialised cercariae do not possess a pointed or tapering anterior end, buccal cavity stylet or penetration glands. They, therefore, are equipped with none of the systems normally associated in cercariae with the ability to penetrate tissue barriers. Indeed, their highly flattened, laterally expanded head seems particularly poorly adapted for any such behaviour. The cercariae nonetheless emerge from M.tuberculata snails within 20 minutes of a reduction in external illumination which initiates their emergence migration. Although in Chapter 3 it was speculated that enzymes from the cercarial gut might be implicated in the production of an emergence pore, there is to date no direct evidence for such a mechanism.

The present study has provided new information in this area of transversotrematid cercarial biology but it has been hampered by the requirement to utilise serial sectioning methodology. Only by this means was it possible to map the locations of cercariae and rediae within snails subsequent to an illumination change. Serial sectioning proved to be an efficient yet laborious technique. With it, all larvae in a snail could potentially be located, but at the cost of a very great expenditure of time. As a result it was impossible to utilise as large a number of replicate snails as would have been desirable. Now that the rectal sinuses have been identified as the key region from which emergence appears to occur, it is possible

that future experiments could avoid the need for cercarial sectioning. It should be feasible to dissect away the rectal region from experimental snails and to tease out and count larvae contained in this area. This technique would provide a very rapid estimate of cercarial movements from the digestive gland and a much wider range of experimental designs, with high replication, would immediately become possible.

Other experimental approaches might also generate data on the mechanisms of T. patialense cercarial emergence behaviour. The hypothesis that intestinal digestive enzymes of the cercariae would be implicated in emergence pore production could be tested by histochemical techniques for, for instance, acid phosphatases in or around cercariae within the rectal sinuses. TEM examination of cercariae in this location might also provide insights into the possible mechanisms of parasite-induced pathogenesis.

In the Discussion section of Chapter 4, the possible adaptive significance of the observed circadian pattern of T. patialense cercarial emergence was analysed. It was concluded that when the temporal patterning of snail and fish host behaviour cycles were considered, the observed circadian cercarial output probably served to enhance transmission success. The experiments described in Chapter 4 provided a picture of the interactions between external illumination regimes and the pattern of cercarial emergence. Some definite indications of an endogenous circadian control system or systems were also obtained. Despite this information and, as is the case for all previous findings in this area (see, for instance, Luttermoser, 1955 ; Wagenbach & Alldredge, 1972; Theron, 1975), it has proved impossible to deduce whether the rhythmical attributes of emergence were snail- or parasite-centred or a combination of both. It appears that only a much more elaborate experimental design than those used here would be capable of answering a question of this type. Two approaches are, at least theoretically, plausible. Firstly, if rediae and the cercariae that develop from them could be maintained for even relatively short periods, in conditions of in vitro culture, the influence of snail physiology would be removed. If it could be shown in such circumstances that cercarial

behaviour was rhythmically related to illumination cycles, the potential for a parasite-centred circadian rhythm of emergence would have been demonstrated.

Secondly, intermolluscan larval transplantation might provide useful clues. If cercariae from a Melanoides on one illumination regime could be transplanted into the haemocoelic system of another Melanoides on a regime 12 hours out of phase, the resulting cercarial behaviour would be highly informative. If the cercariae emerged when they should have in respect of the donor snail, it would suggest a parasite-centred control system was operating. If, on the other hand, emergence was at the time suggested by the recipient snails illumination cycle, a snail-centred system would be more likely. Although intermolluscan larval transplantation has not as yet been attempted with transversotrematids, it has proved technically possible in a range of digenean families (see, for instance, Dönges, 1971) and might be expected to succeed.

Chapters 5, 7 and 8 of this thesis have presented data on the structural organisation of the neural system and muscular effectors of T. patialense cercariae from a number of viewpoints. The most useful have been the analysis of the general disposition of nerve tracts made possible by histochemical,light microscopical techniques and the ultrastructural details demonstrable by TEM methodology. Chapter 6 has supplemented these largely structural sets of information with data on the effects of pharmacological agents on cercarial activity.

If all this observational and experimental evidence is placed alongside previous findings on the cercarial behaviour of T. patialense (Anderson & Whitfield, 1975; Whitfield, Anderson & Moloney, 1975; Whitfield, Anderson & Bundy, 1977) it is possible to attempt a synthesis concerning the patterns of neural control of muscular activity that exist in these larvae.

We are dealing here with two interconnected nervous systems, those of the cercarial head and tail respectively. Much that has been discovered in the present research programme has confirmed the earlier indications (Whitfield, Anderson & Moloney, 1975; Whitfield, Anderson & Bundy, 1977) that the tail is, at least in propulsive terms, a potentially independent organ system.

The whole tail complex (stem, arm processes and furcae) is shed late in the attachment process and has then been observed to continue with rapid lateral beating for some time after shedding (Whitfield, Anderson & Moloney, 1975). The results reported in Chapter 6 have shown that experimentally removed tails behave in the same fashion. In these experiments control tails remained active for in excess of 100 minutes. Their coordinated swimming activity for this period while disconnected from the nervous system and receptors of the head dramatically indicates the tail's intrinsic power of coordination and control. The cercarial tail is a "disposable" organ, used only in the dispersal and host finding phase of the cercarial life span, yet it evidently contains an important and partially self-regulating nervous system of its own.

The relative independence of the tail is emphasized by the marked differences which were apparent in the responsiveness of heads and tails of T. patialense cercariae to different pharmacological agents (physostigmine, adrenaline, atropine, nicotine).

It has been suggested before (Whitfield, Anderson & Bundy, 1977) that the ability of the naturally detached tails of T. patialense cercariae to continue with rhythmic swimming activity might indicate the presence of a neural pace-maker region in the tail. The results of this thesis on artificially separated tails do nothing to make this idea less tenable.

It would appear that the anterior and posterior nerve masses of the tail stem are the most likely candidates for a pace-maker role of this type. They are located in close proximity to the striated musculature of the tail and it seems very likely that nerve processes from the masses pass to the muscles. It must be emphasized, however, that there exists, at present, no direct evidence for a neural pace-maker. The continued patterned contractions of the tail musculature after detachment could be the result of other control system types. Myogenic activity in the striated muscles, for instance, could initiate such contractions and the pace-maker-like control could itself reside in the muscle cells.

In vertebrate hearts the cardiac muscle itself has the inherent property of rhythmicity in that the muscle fibres contract and relax alternately in a rhythmical manner. This property is developed to the greatest extent in a region known as the sino-atrial node (SA node) which is situated in the wall of the right atrium.

This SA node originates each heart beat and it is known as a pace maker. No nerves are involved in the spread of a contraction wave through cardiac muscles (Green, 1977).

In intact T. patialense cercariae continuous swimming does not occur. While in the water column, the larvae alternate between swimming, dropping and resting behaviour (Whitfield, Anderson & Bundy, 1977), while on the fish host a very complex attachment behaviour sequence is initiated (Whitfield, Anderson & Moloney, 1975). In both of these circumstances swimming activity of the tail is temporarily suspended while other activities (or none) occur. The achievement of these aspects of the behavioural repetoire require the presence of the head and it is reasonable to assume that it is the central nervous system in the head which controls such behaviour. In addition, given the intrinsic "continuous-activity" status of the tail, complex behaviour control must require centrifugal inhibition as well as particular excitatory signals from the cerebral ganglion or other parts of the central nervous system.

The pharmacological investigations and histochemical results of the present body of work are able, once again, to shed some light on the mechanisms of such central nervous system-generated inhibitions. The drug exposure experiments suggested strongly that one or more catecholamines must be acting as inhibitory transmitters in T. patialense cercariae. It is reasonable to assume that the nerve pathways containing them are at least partly responsible for the inhibitory aspects of complex behaviour. The results have also shown that it is likely that the cell bodies of such neurones, (i.e. catecholamine-containing ones) are only present round the periphery of the cerebral ganglion and the main branches of the central nervous system. This helps to confirm the idea that it is the central nervous system of the head that controls complex behaviour.

The results of the present study will hopefully provide a framework for more detailed and in some cases more technically sophisticated analysis of the neural and muscular organisation of these interesting larval parasites. Biochemical techniques could be used to determine the nature and concentrations of neuro-transmitters present in the parasites. Spectrophotofluorametrical

methods could be particularly useful in this respect. Using scanning electron microscopy and transmission electron microscopical techniques it should be possible to identify and characterise all the surface receptors of T. patialense cercariae and to follow some of their neural connections with the central nervous system. It is interesting to consider the fact that, despite the large number of publications on the surface receptors of parasitic flatworms, in not one case has the location of the nucleated cell body of the sensory cell been discovered.

These biochemical and structural analyses could be usefully augmented by further physiological studies. The drug exposure experiments of the present research programme could be extended so as to examine excitatory influences. This would require the analysis of drug-related behaviour changes in cercariae that were not stimulated before the observation period. In addition, it would be extremely useful to be able to make electrophysiological recordings from cercarial nerves and muscles with and without externally applied pharmacological agents.

To date, such recordings have rarely, if ever, been attempted (Prior & Uglem, 1979). With a large cercaria like that of T. patialense, however, the use of suction and/or microelectrodes should be possible. The detailed structural information on the central nervous system and muscles provided by this study would make the positions of such electrodes a directed rather than a random procedure.

Finally, although the technical difficulties would be great, the highly ordered nature of the caudal striated muscles suggests that X-ray diffraction studies could be attempted. Low angle X-ray diffraction on living material might enable one to examine changes in the patterns of cross-striations and myosin/actin overlap in extended and contracted muscles.

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Plate 3.1 Sagittal section through the body of the snail Melanoides tuberculata

A. Cercariae (c) and rediae (r) within the haemocoelic spaces of the digestive gland.
(dgl) digestive gland lobule.

B. Portion of the digestive gland at higher magnification to show the presence of cercariae (c) and rediae (r) within the haemocoelic space.

dgl

c

r

0.4 mm

A

c

r

0.1 mm

B

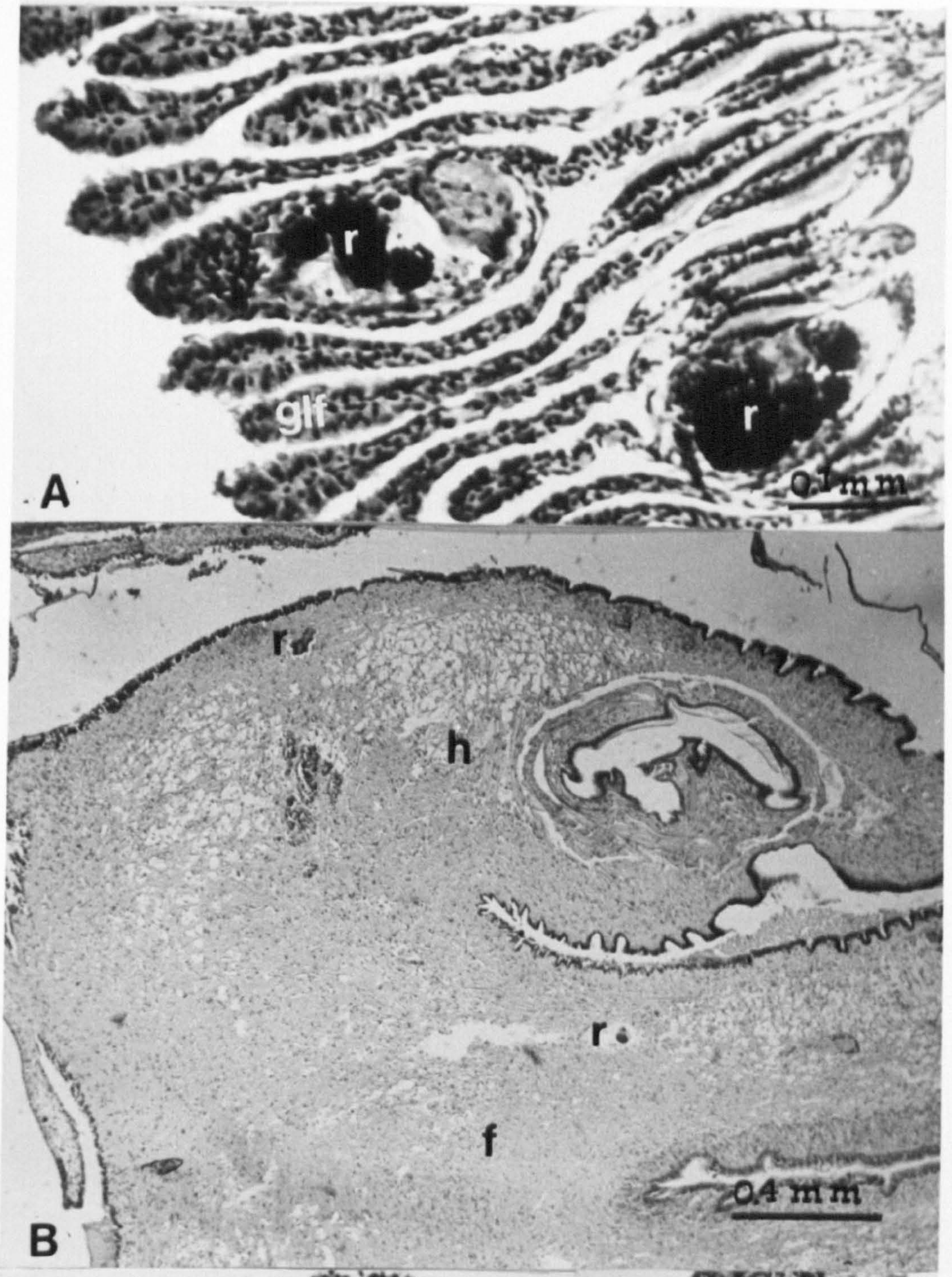
Plate 3.2 Sagittal section through the body of the snail Melanoides tuberculata

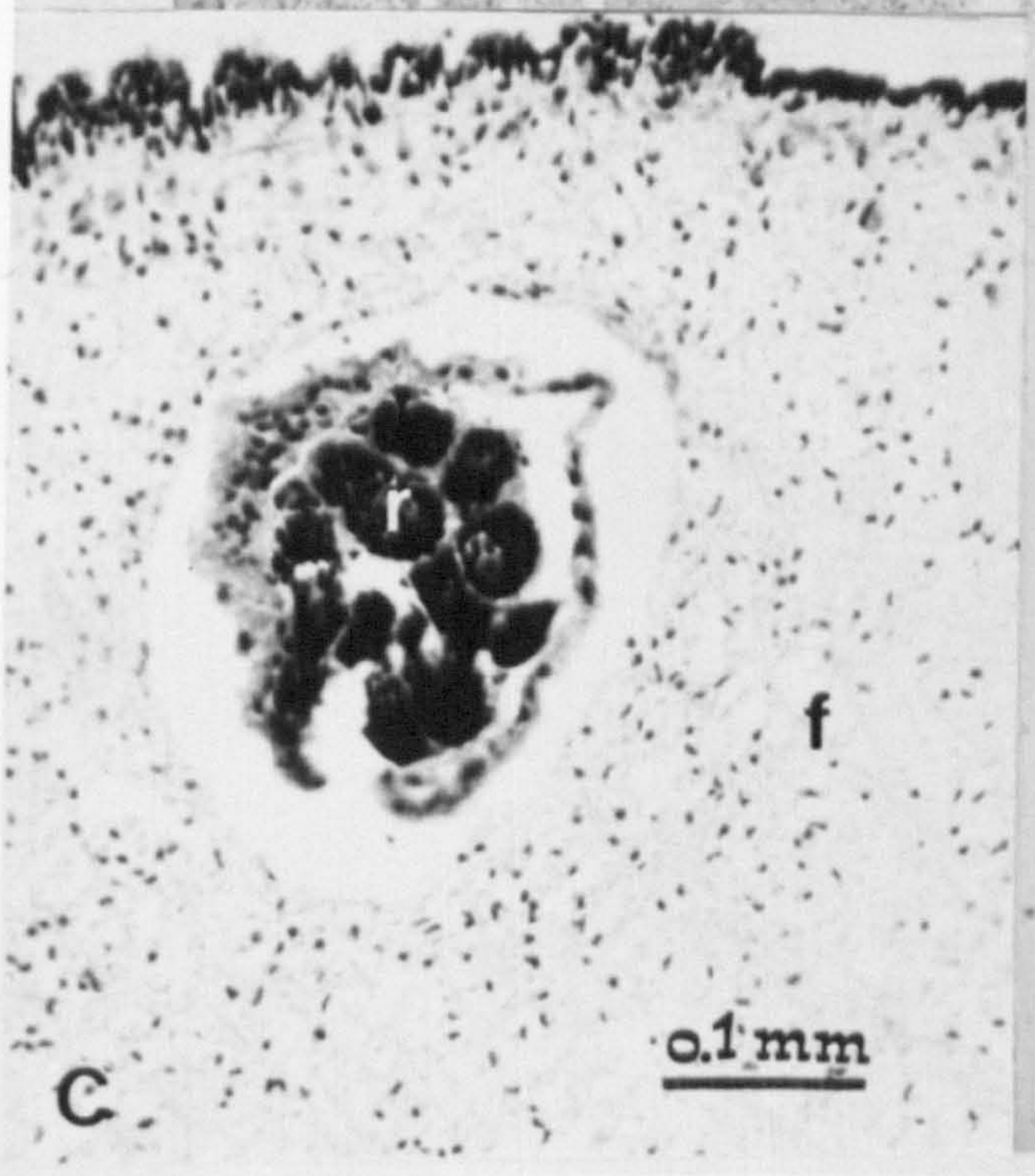
A. Rediae (r) within the gill filaments (gf)

B. Rediae (r) within the head (h) and foot (f)

C. Redia (r) within the foot (f)

D. Redia (r) within the mantle sinus (mts)


r
glf
r
0.1 mm
A
r
h
r
f
0.4 mm
B


r
f
0.1 mm
C

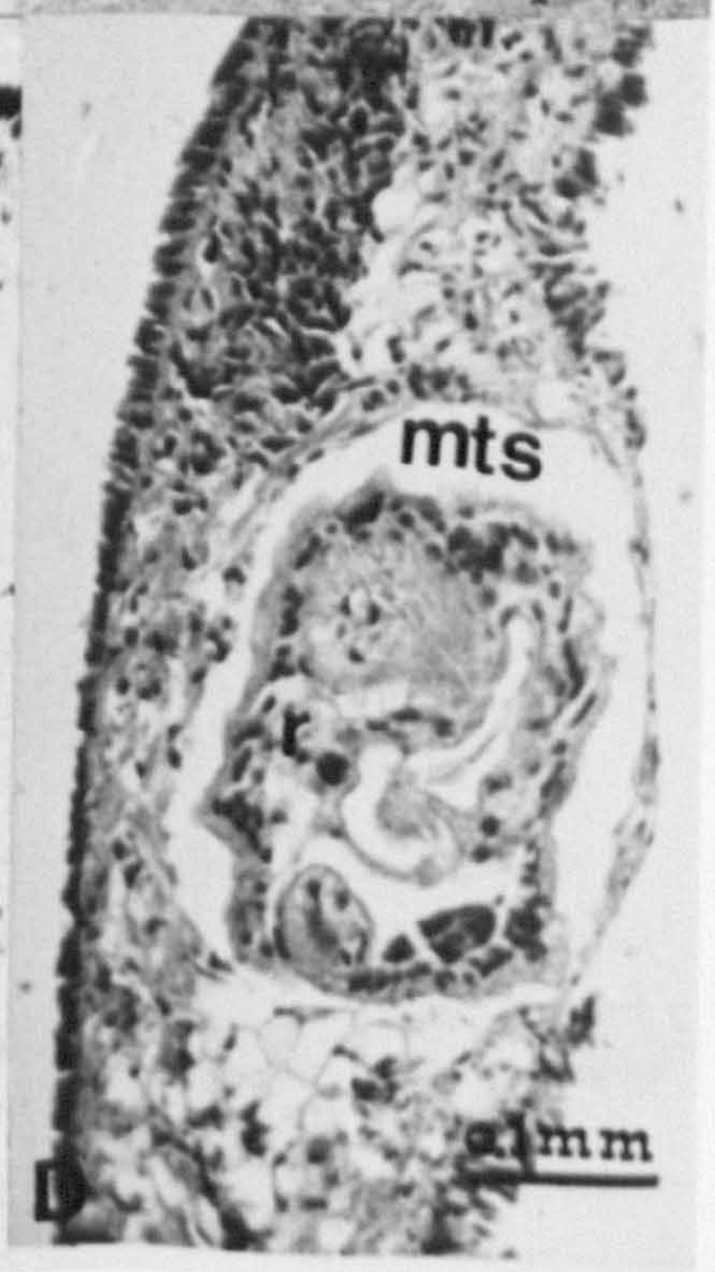

mts
t
0.1 mm
D

Plate 3.3 Sagittal section through the body of the snail Melanoides tuberculata

A. Cerariae (c) and rediae (r) within the visceral sinus (vs) in:intestine

B. Cercariae (c) within kidney sinus (ks)


r
VS
in
c
VS
c
0.1 mm
A
ks
c
0.1 mm
B

Plate 3.4 Sagittal section through the body of the snail Melanoides tuberculata

A. Cercaria (c) lying free in the rectal sinus (rcs) adjacent to the rectum whose lumen (rc) contains faeces (f)

B. Cercariae (c) within the rectal sinus (rcs) near the anus (an)

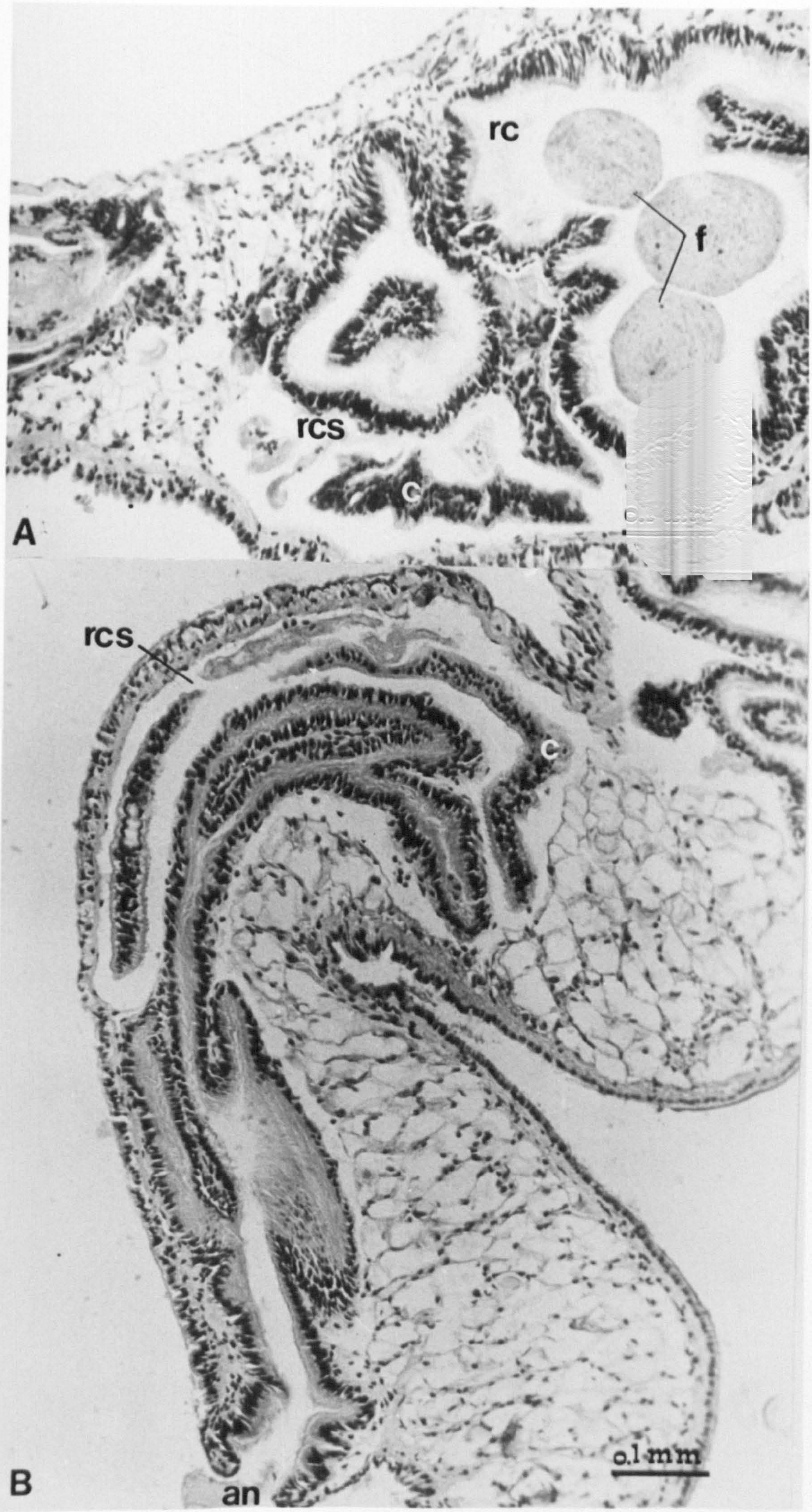

rc
f
rcs
c
A
rcs
c
B
an
0.1 mm

Plate 3.5 Sagittal section through the body of the snail Melanoides tuberculata

A. Cercariae (c) within the mantle sinus (mts) beneath the epidermis

B. Cercaria (c) in the mantle cavity (mtc)

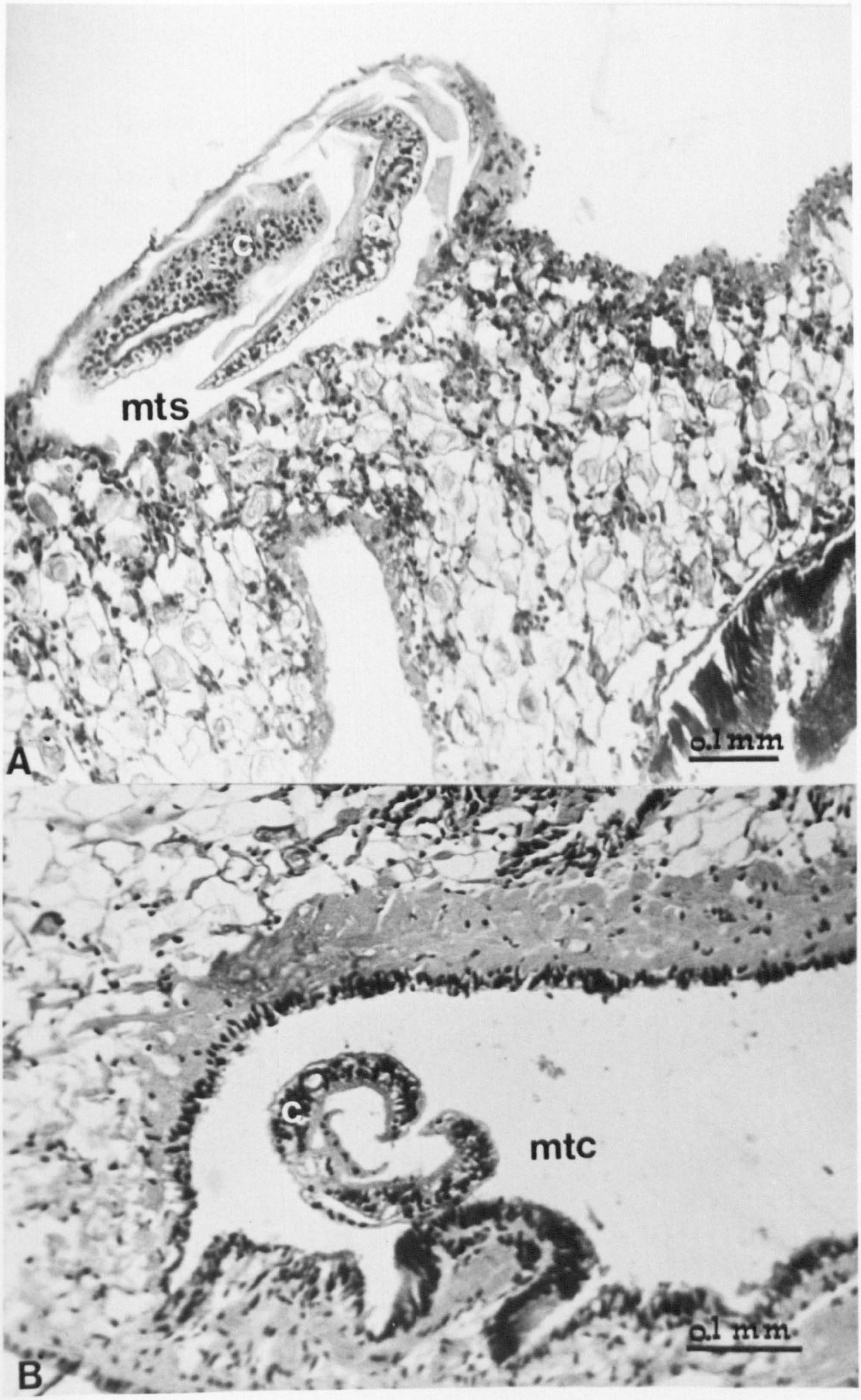

C
mts
0.1 mm
A
C
mtc
0.1 mm
B

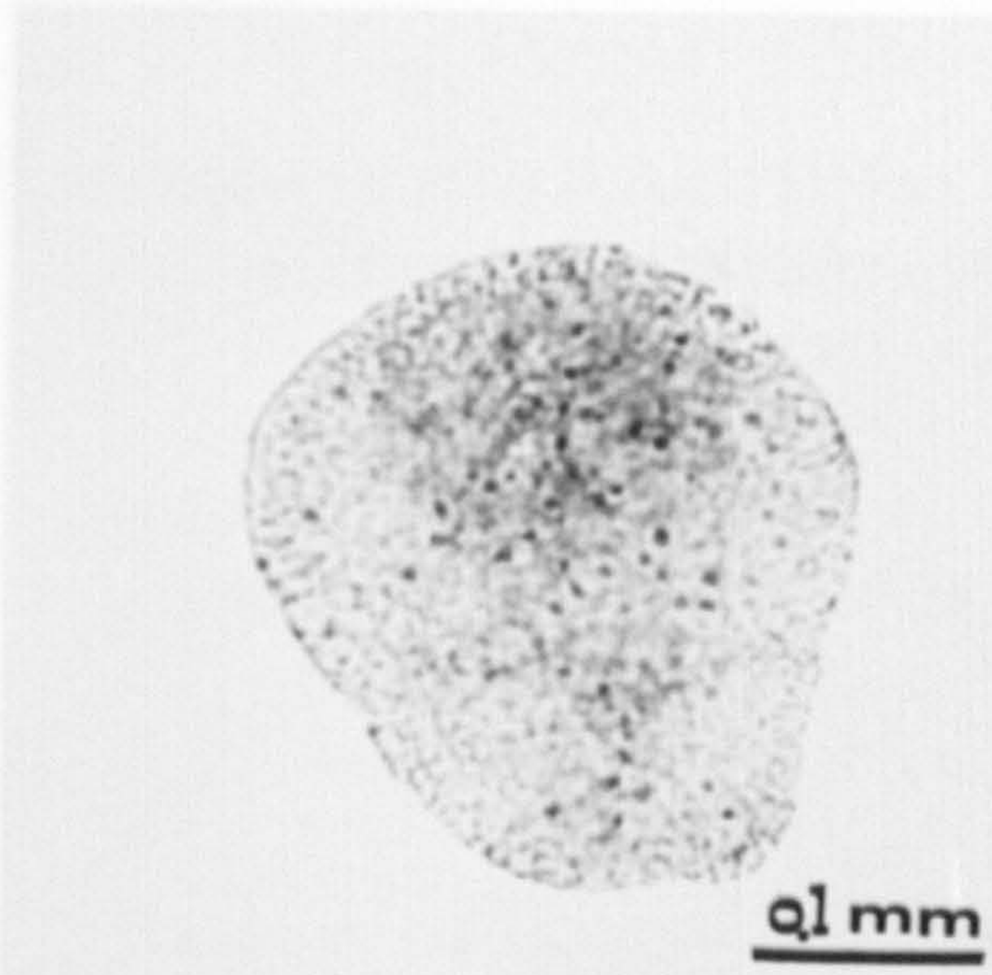
Plate 5.1 <u>Micrographs of intramolluscan development stages of cercariae showing stages in the development of the nervous system (5-bromoindoxyl acetate method)</u>

(A) Early type 1 cercaria with no evidence of nerves

(B) Late type 1 cercaria with no evidence of nerves.

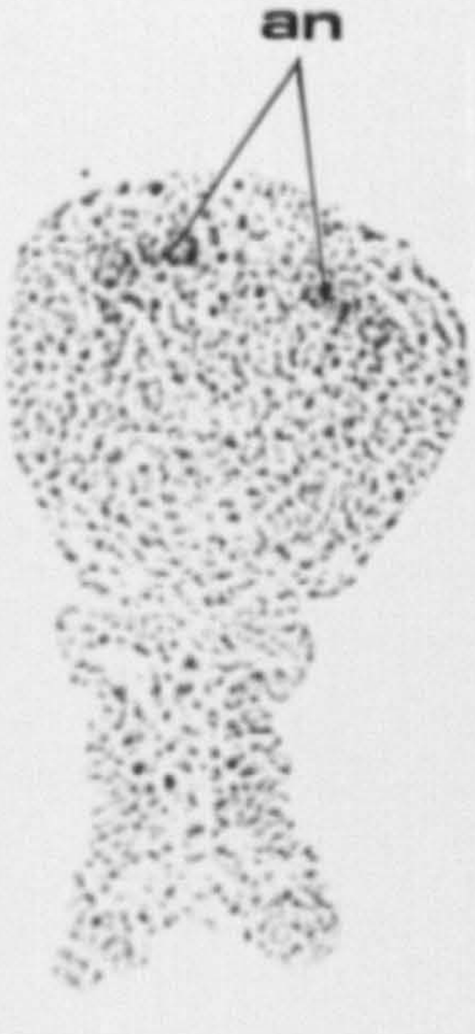
(C) Late type 2 cercaria showing a bilateral anterior concentration of positively staining primordial neural tissue (an)

(D) Late type 2 cercaria showing primordial longitudinal nerve tract in the middle of the tail (ln)

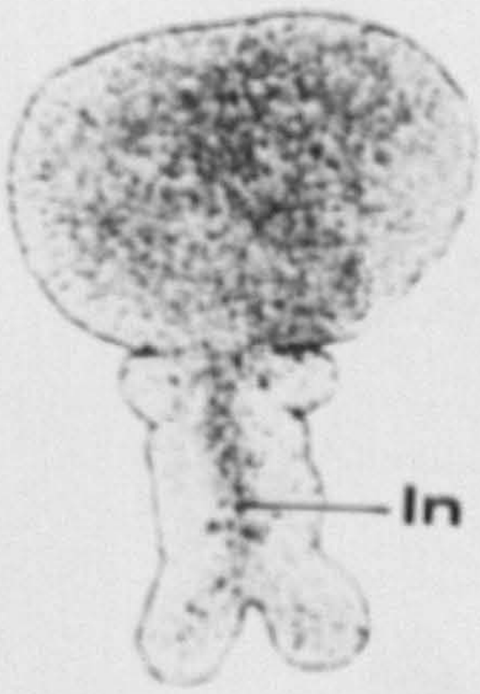


A

B



C



D

Plate 5.2 <u>Micrographs of the developing and mature free-living cercariae showing the structure of the nervous system (5-bromoindoxyl acetate method)</u>

(A) Type 3 developing cercaria with two anterior concentrations of neural tissue (an), a longitudinal tract passing into the tail (lt). Faint traces of primordial laterally directed nerve branches are also visible.

(B) Type 4 mature free-living cercaria showing the principal components of the nervous systems of the head and tail (compare with Figure 5.4)

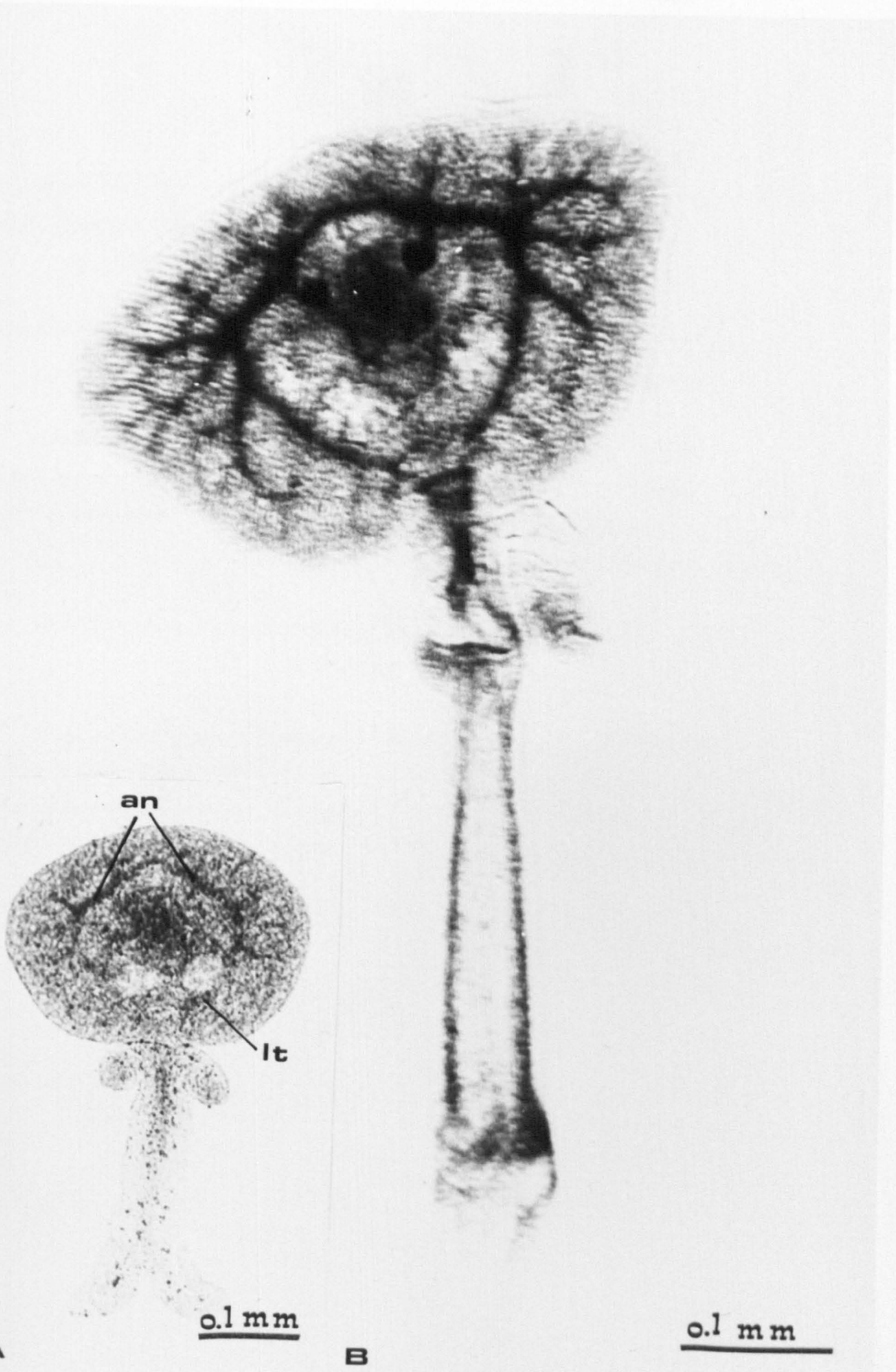

an
lt
0.1 mm
A
B
0.1 mm

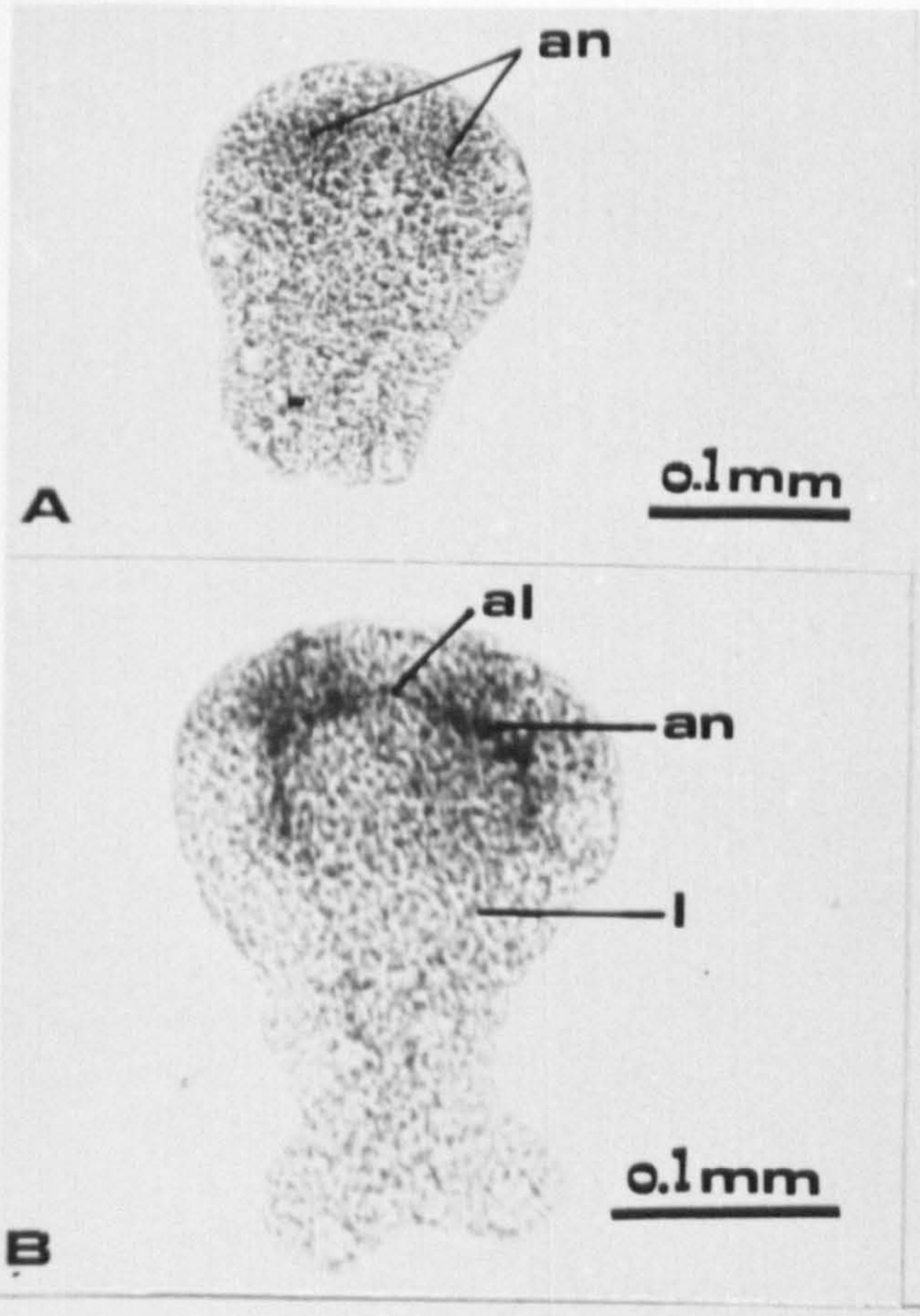
Plate 5.3 <u>Micrographs of intramolluscan development stages of cercariae showing stages in the development of the nervous system (∝ naphthyl acetate method)</u>

(A) Late type 1 cercaria with an anterior bilobed and symmetrical area of primordial nervous tissue (an)

(B) Early type 2 cercaria with two bilaterally symmetrical anterior concentrations of primordial nervous tissue (an), two longitudinal nerve tracts (l). A fine transverse anterior link is apparent (al)

(C) Late type 2 cercaria with anteriorly directed branches (ab) from the cerebral ganglion areas

(D) Type 3 cercaria: outward-directed branches (ob) have become apparent as have inner branches (ib). A posterior link (pl) between the longitudinal nerve tracts and dense nerve mass (dm) at the body-tail junction are also visible


an
0.1mm
A
al
an
l
0.1mm
B


ab
0.1 mm
C

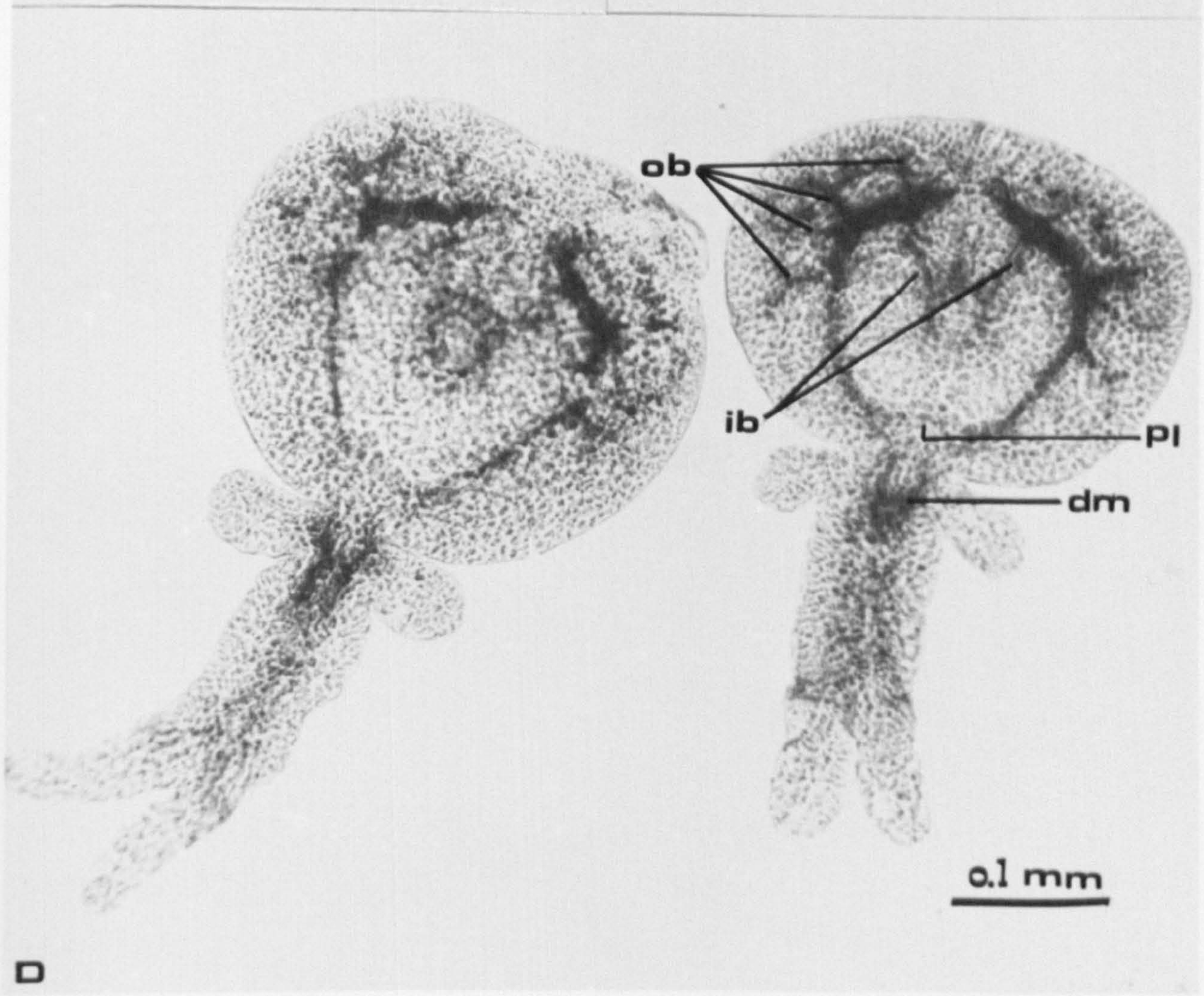
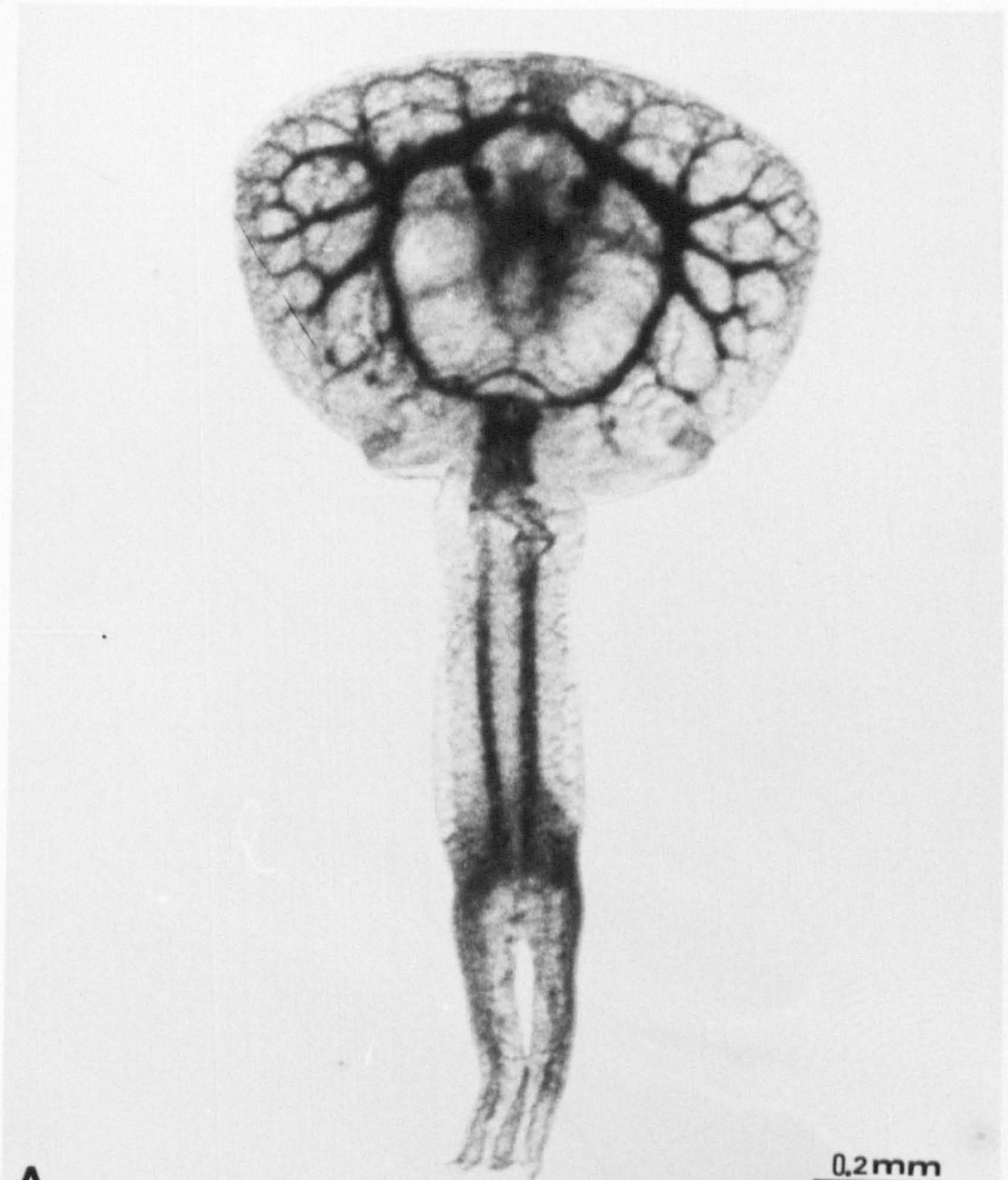

ob
ib
pl
dm
0.1 mm
D

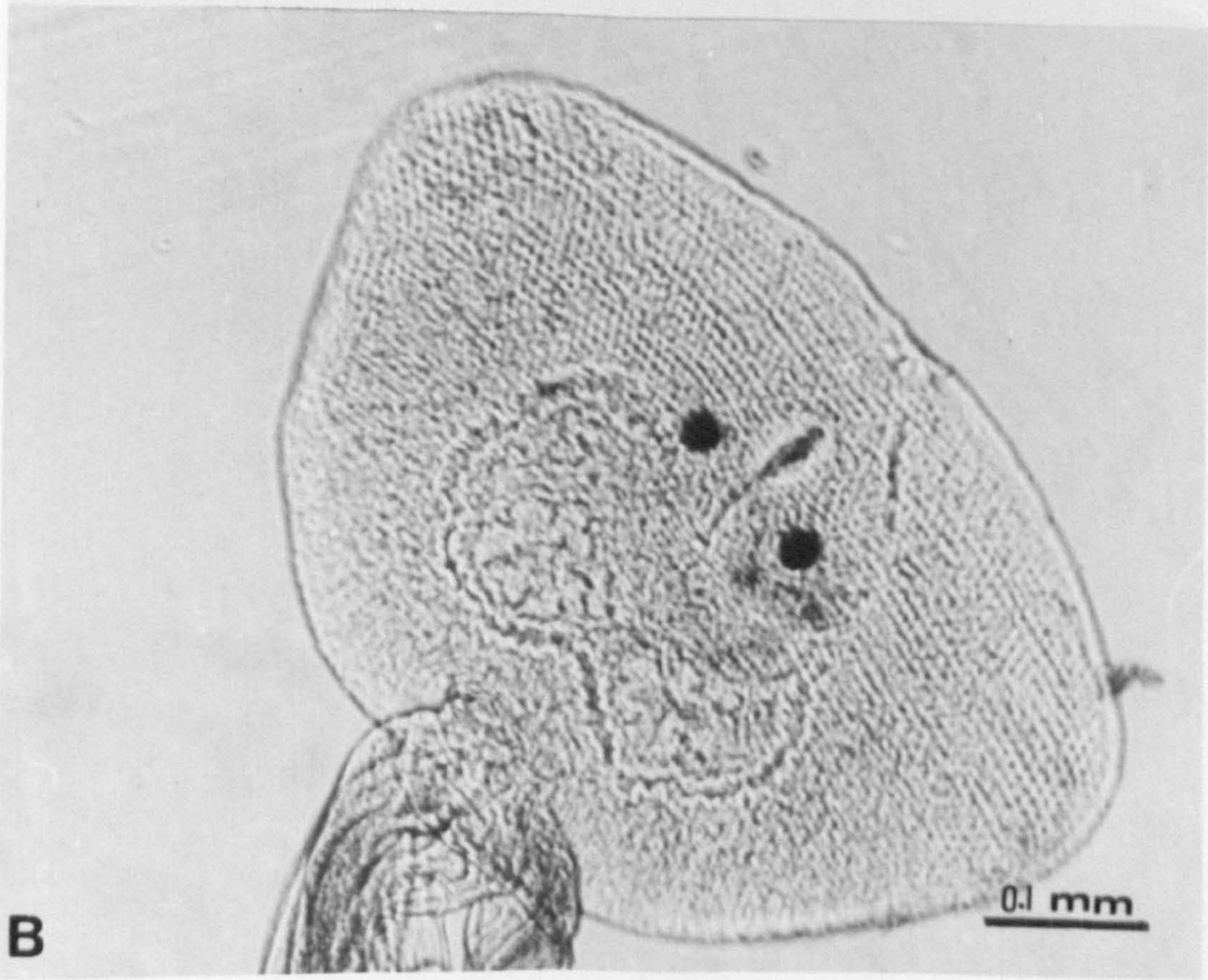
Plate 5.4 <u>Micrographs of mature free-living cercariae after staining with the ∝naphthyl acetate method</u>

(A) Experimental: Type 4 cercaria showing the distribution of esterase activity in the nervous system

(B) Control: Type 4 cercaria showing inhibition of esterase activity in the nervous system when incubated in medium containing 10^{-3}M physostigmine (eserine)



A

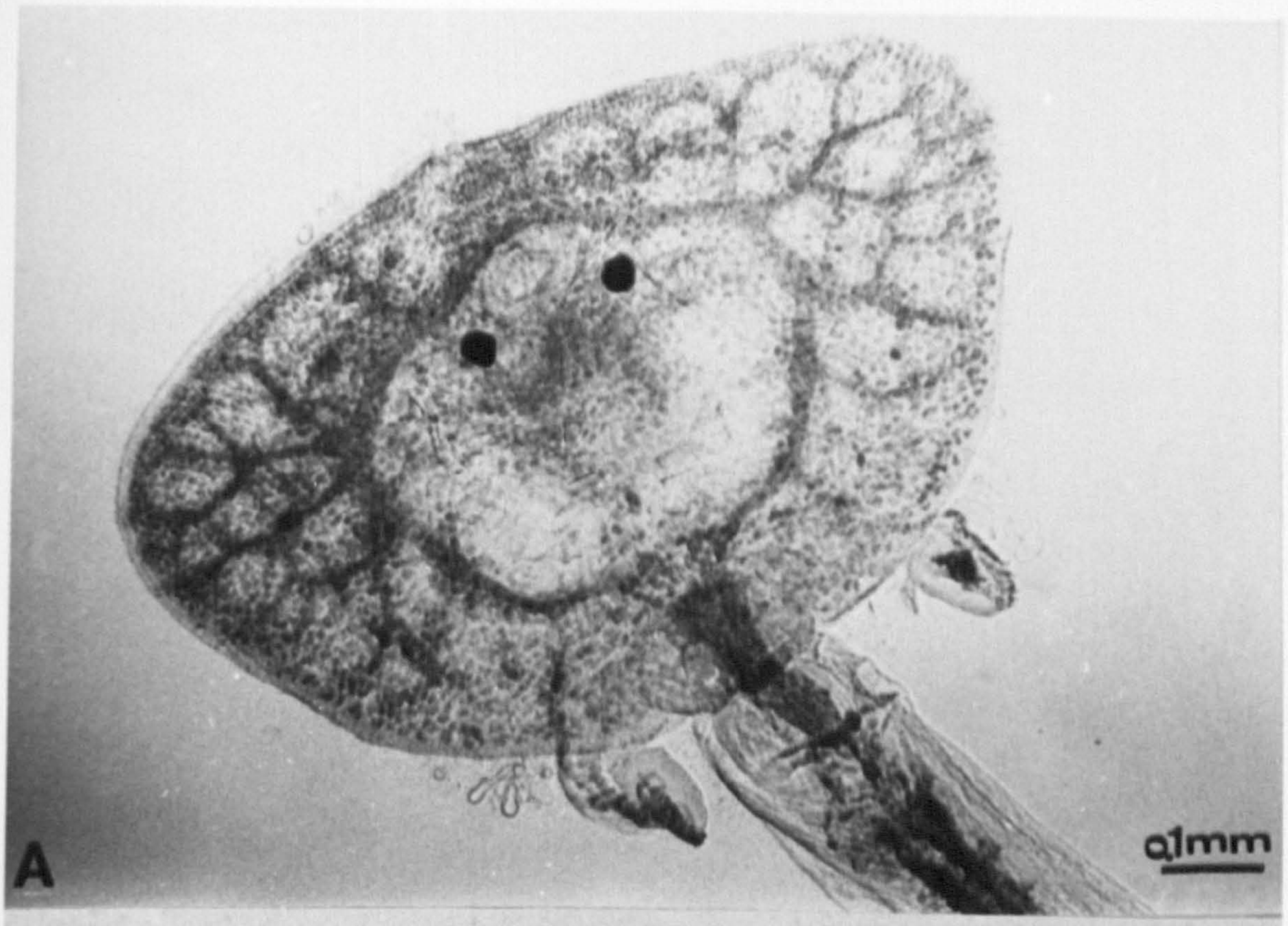


B

Plate 5.5 <u>Micrographs of a mature free-living cercaria with the nervous system visible due to staining with the acetylthiocholine iodide method</u>

(A) Type 4 mature free-living cercaria. Acetylcholinesterase activity distributed throughout the nervous tissues

(B) Control: inhibition of cholinesterase activity after incubation in medium containing 10^{-3}M physostigmine (eserine). No reaction product can be seen


A
0.1mm

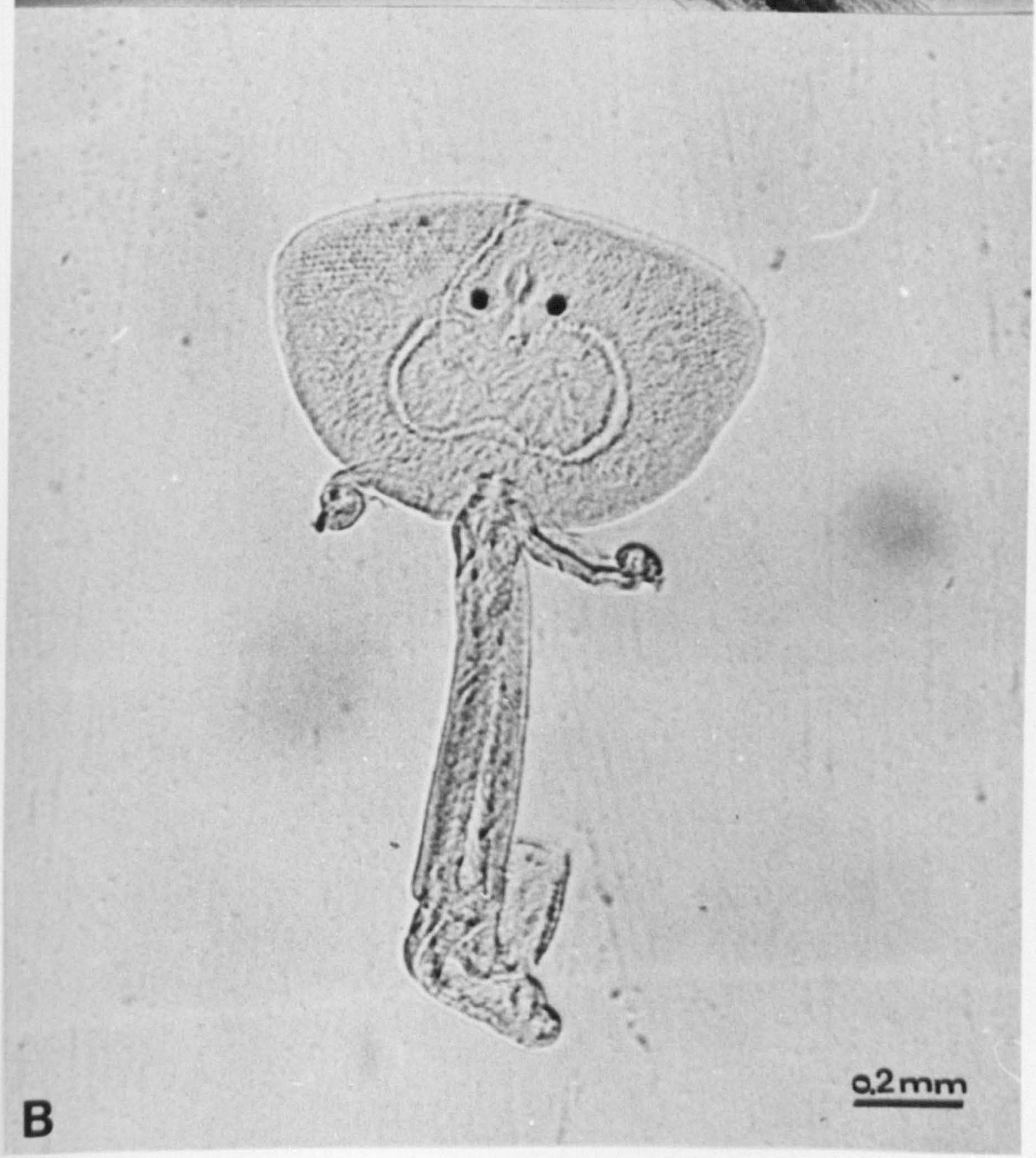

B
0.2mm

Plate 5.6 <u>Fluorescence micrographs showing the distribution of green fluorescence in the nervous structures of cercariae treated with FIF technique</u>

(A) Intense fluorescence in nerve fibre tracts and in fluorescent spots (fs) interpreted as catecholamine-containing nerve cell bodies

(B) Fluorescent fibres (flc) in the longitudinal nerve cords of the tail

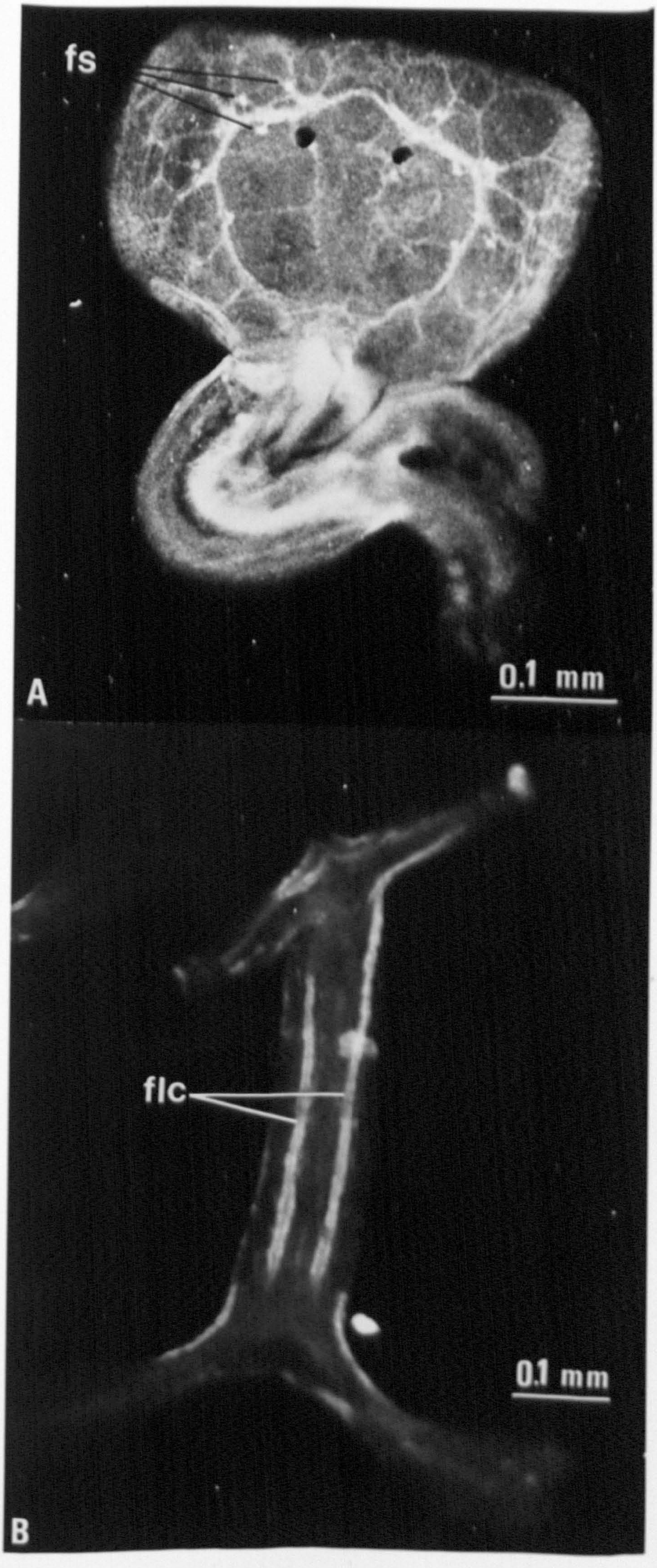

fs
A
0.1 mm
flc
B
0.1 mm

Plate 5.7 <u>Fluorescence photomicrographs showing green fluorescence in the nervous system of cercariae (GA method)</u>

(A) Fluorescence in nerve fibre tracts and in fluorescent spots (fs) interpreted as catecholamine-containing nerve cell bodies.

(B) Fluorescence (flc) associated with the longitudinal nerve cords of the tail stem. Note the local thickenings in the fluorescent reaction product.

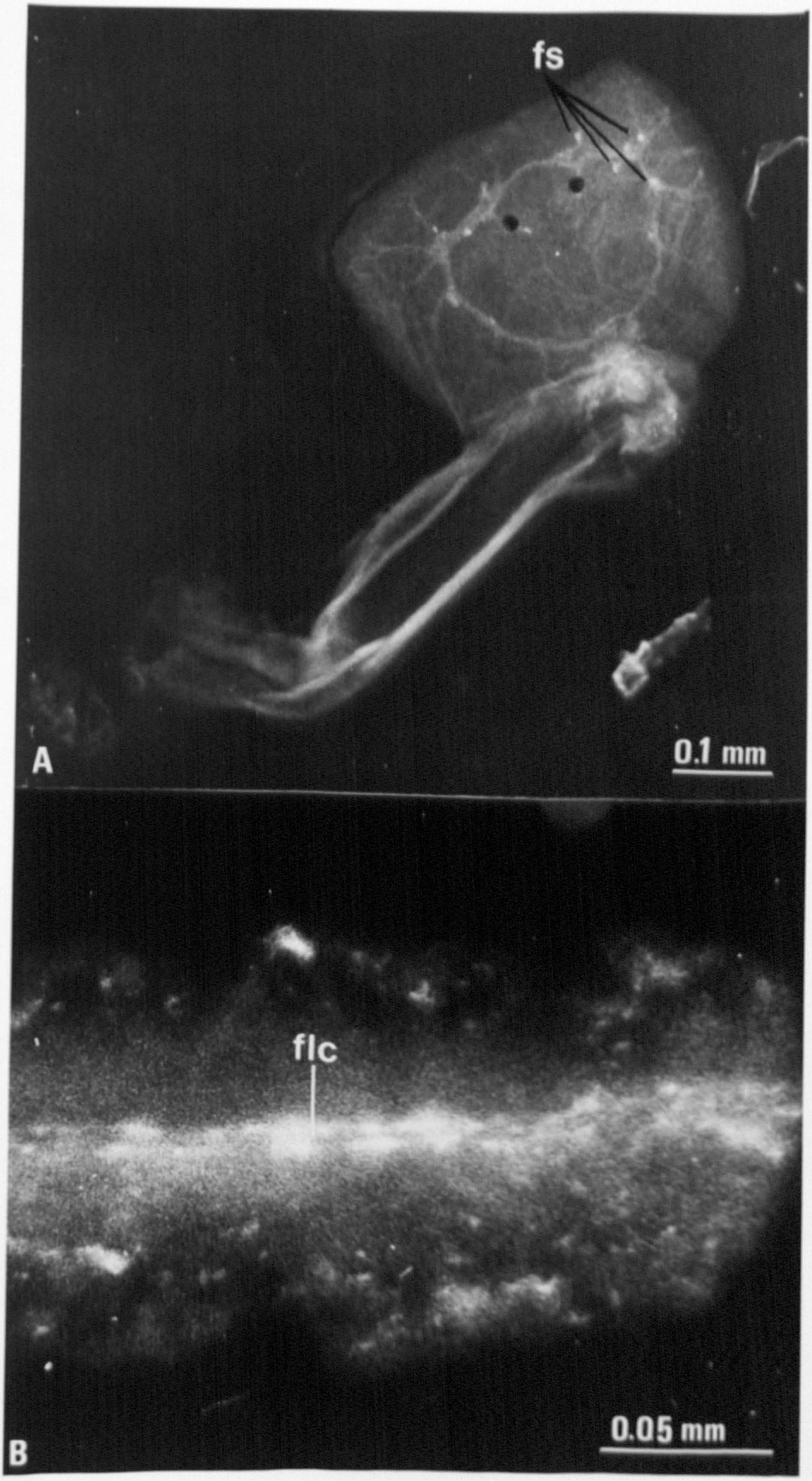

fs
A
0.1 mm
flc
B
0.05 mm

Plate 5.8 Fluorescence photomicrographs of cercariae treated with glyoxylic acid method and in the absence of paraformaldehyde vapour and glyoxylic acid

(A) Experimental (GA method):
Fluorescence in the male genital organs (mgo)

(B) Control (FIF method: no formaldehyde):
There is a marked reduction in the fluorescence associated with the nervous system

(C) Control (GA method: no glyoxylic acid):
There is a marked reduction in the fluorescence associated with the nervous system

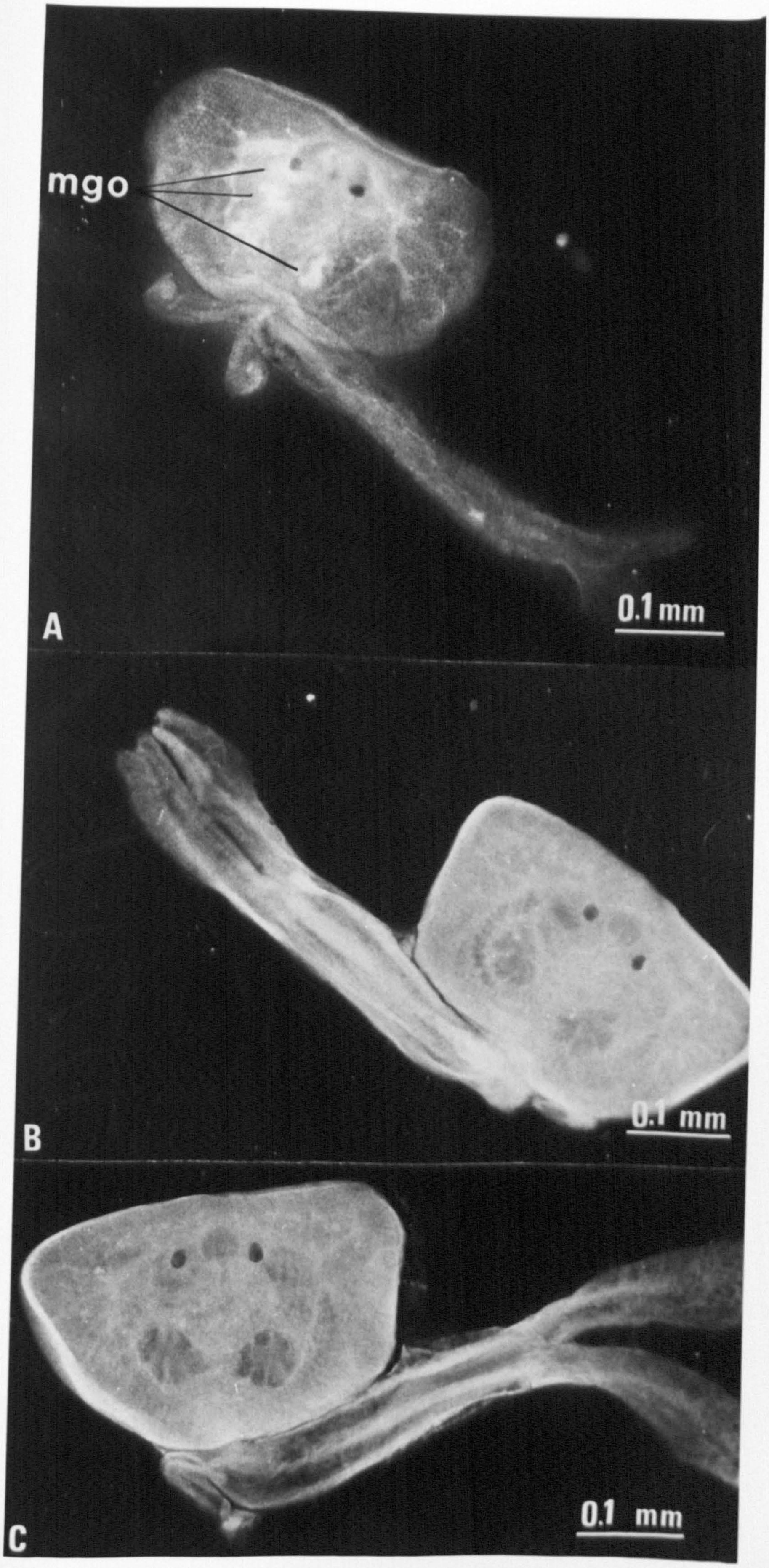
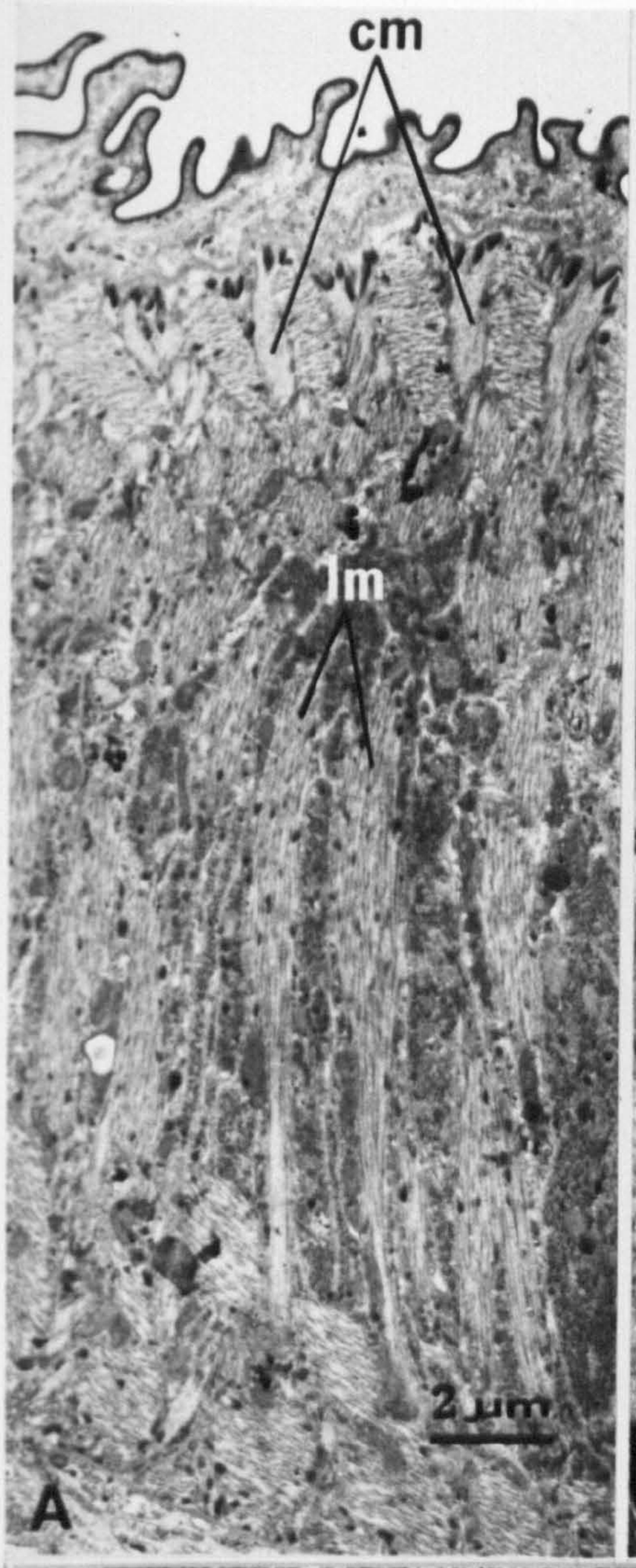

mgo
0.1 mm
A
0.1 mm
B
0.1 mm
C

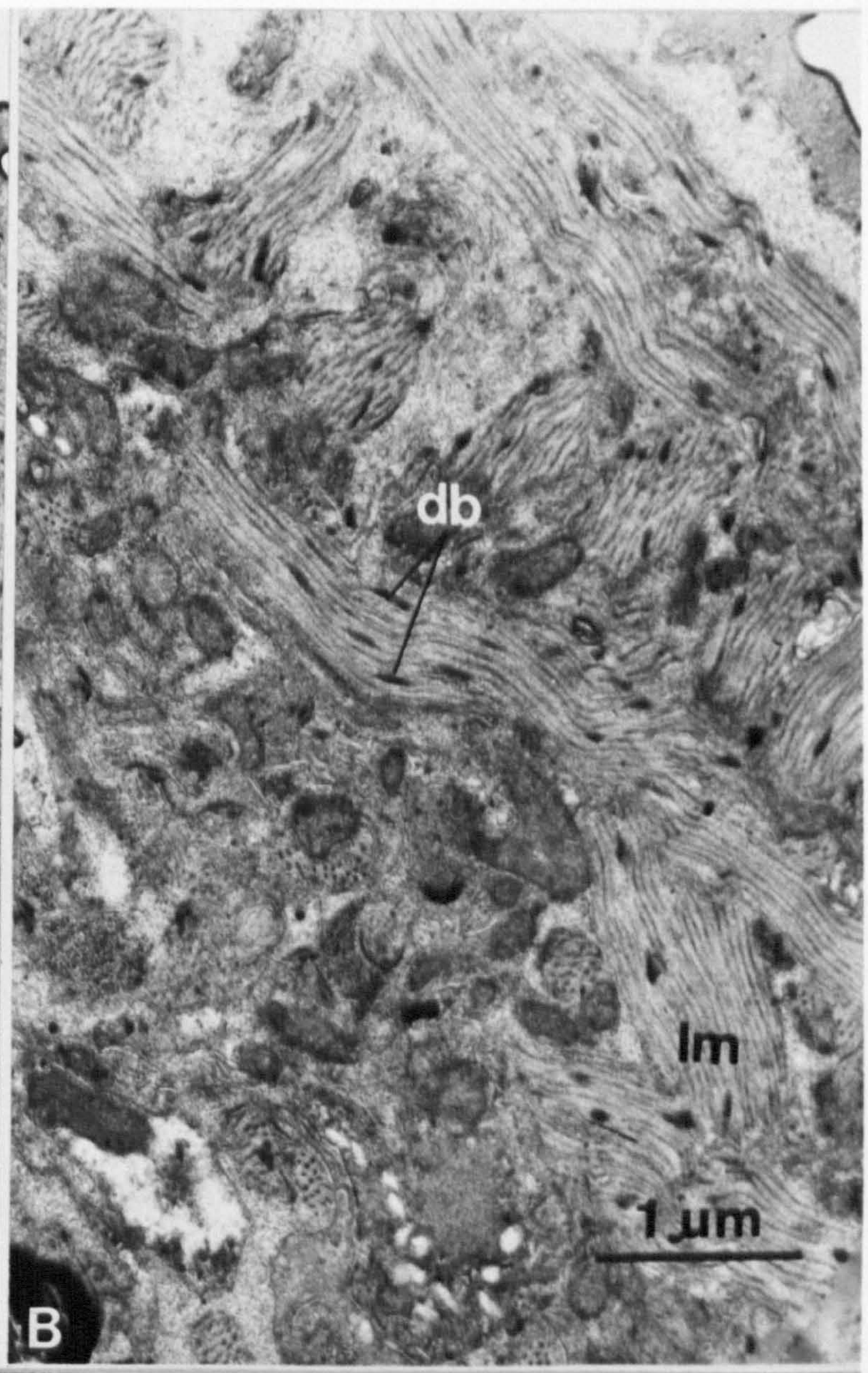
Plate 7.1 Transmission electron micrographs of body wall musculature of the cercarial head.

(A) Circular non-striated muscle fibres (cm) and longitudunal non-striated muscle fibres (lm) beneath the surface tegument.

(B) Longitudinal non-striated muscle fibres (lm) arranged in three or four sets of obliquely distributed fibres. They contain thick and thin myofilaments and dense bodies (db).

(C) Longitudinal non-striated muscle fibres in cross section associated with nervous elements (ne). Cisternae of sarcoplasmic reticulum (sr) closely approximate the sarcolemma. Dense bodies (db) are associated with shallow infoldings of the sarcolemma.


cm
lm
2 µm
A


db
lm
1 µm
B

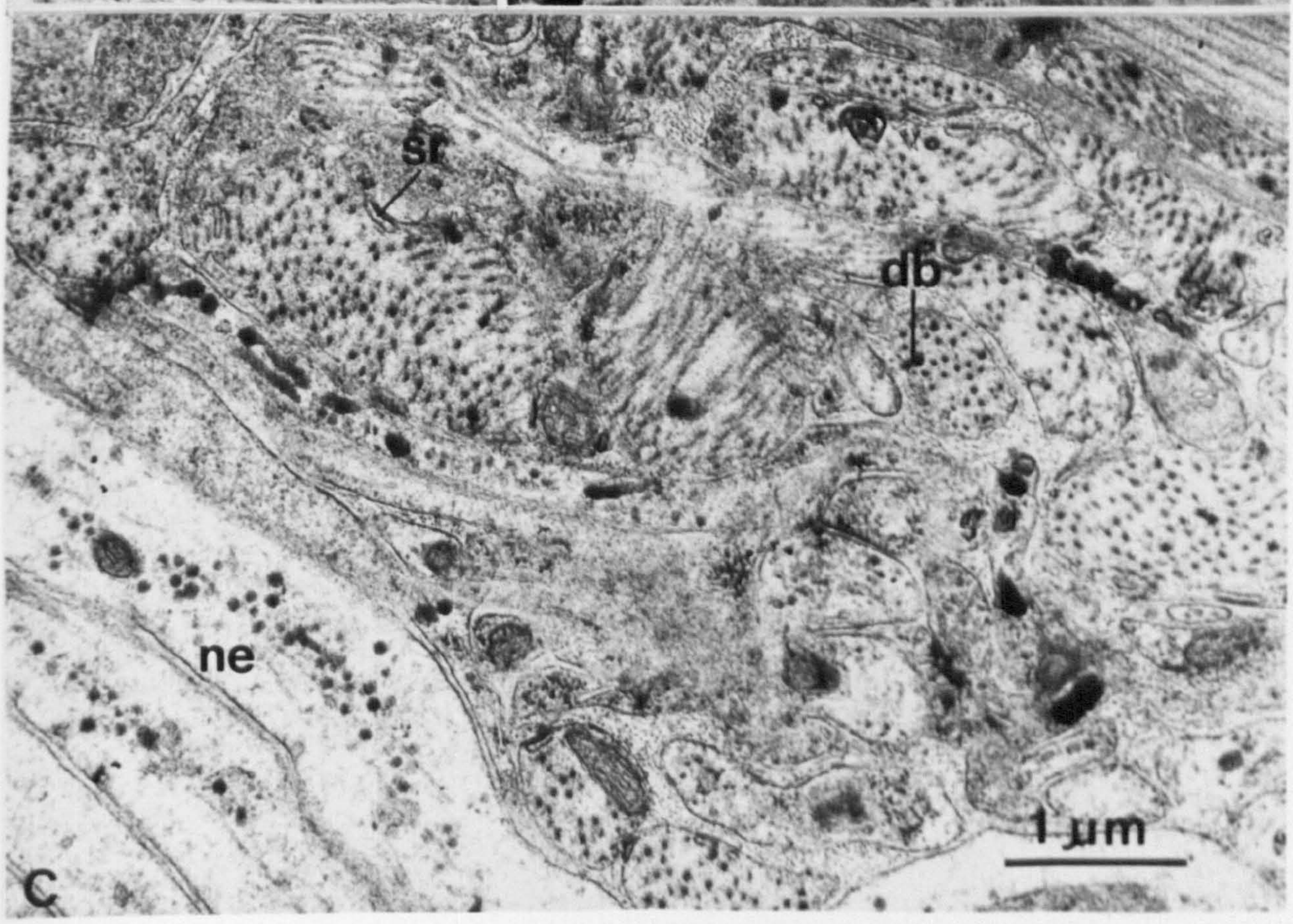

sr
db
ne
1 µm
C

Plate 7.2 Transmission electron micrographs of caudal non-striated circular muscles of the tail stem.

(A) Transverse section through the tail stem of a cercaria showing a circular non-striated muscle fibre sectioned longitudinally (cm). The myofibre contains thick (tk) and thin (tn) myofilaments with dense bodies (db), mitochondria (m), and glycogen granules (g).

(B) Longitudinal section of the tail stem with transverse profiles of circular non-striated muscle fibres (cm).
db: dense bodies attached to the basal lamina and basal membrane of the tegumentary distal cytoplasm
sr: cisternae of sarcoplasmic reticulum

(C) Transverse section through the circular non-striated muscle fibre showing the thick myofilaments (tk) and thin myofilaments (tn), cisternae of sarcoplasmic reticulum (sr) and mitochondria (m)

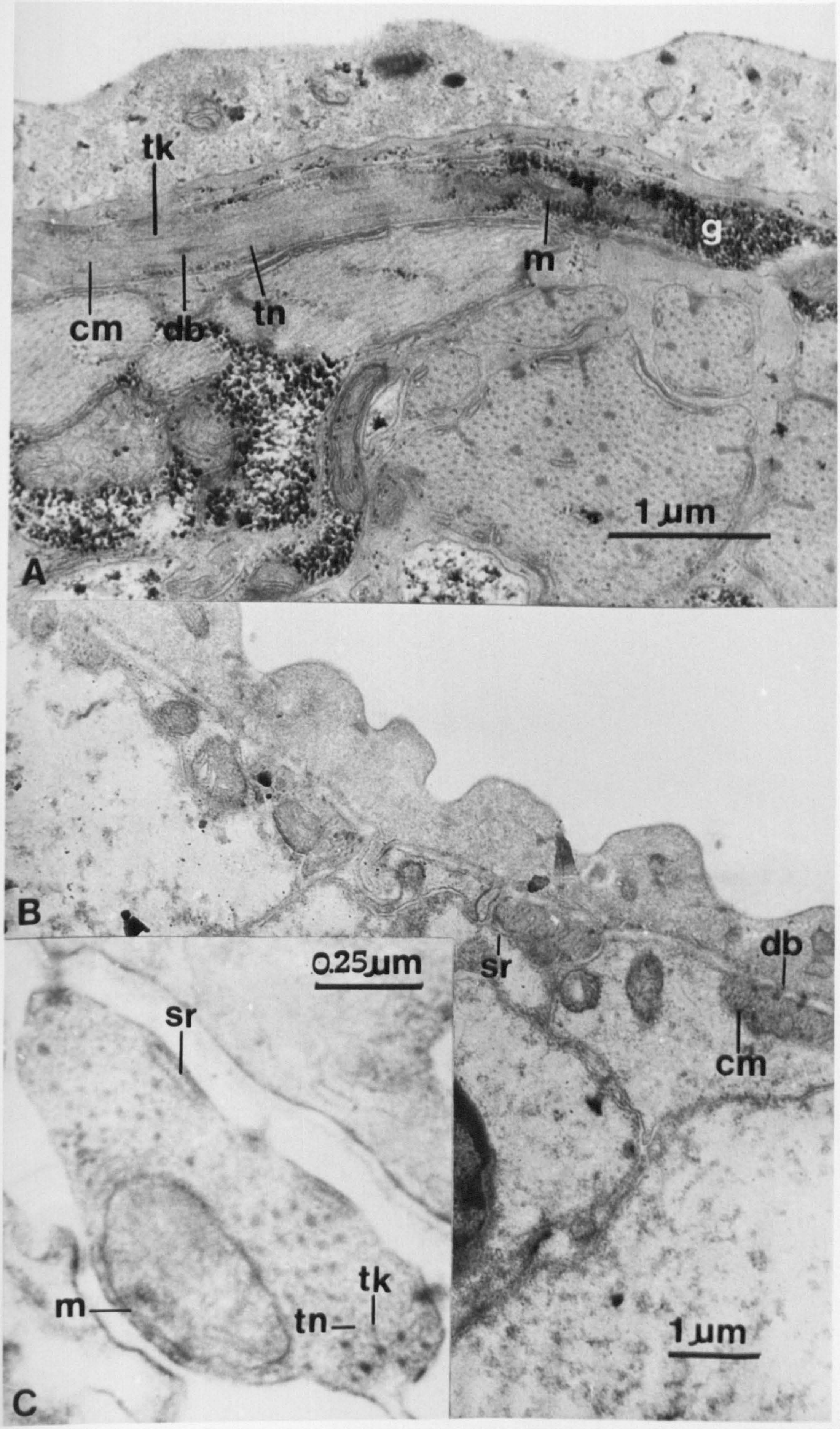

tk
cm
db
tn
m
g
1 µm
A
B
0.25µm
sr
sr
db
cm
m
tn
tk
1µm
C

Plate 7.3 (A) Transmission electron micrograph of a transverse section of a longitudinal non-striated tail stem muscle fibre showing thick (tk) and thin (tn) myofilaments, dense bodies (db) and sarcoplasmic reticulum (sr). m: mitochondria; g: glycogen granules

(B) Transmission electron micrograph of longitudinal section of a longitudinal non-striated tail stem muscle fibre. The intercellular space contains fibrous material (fm). Hemidesmosome-like junctions exist between some of the dense bodies (db) and the fibrous material. Glycogen granules (g) are scattered between myofilaments.

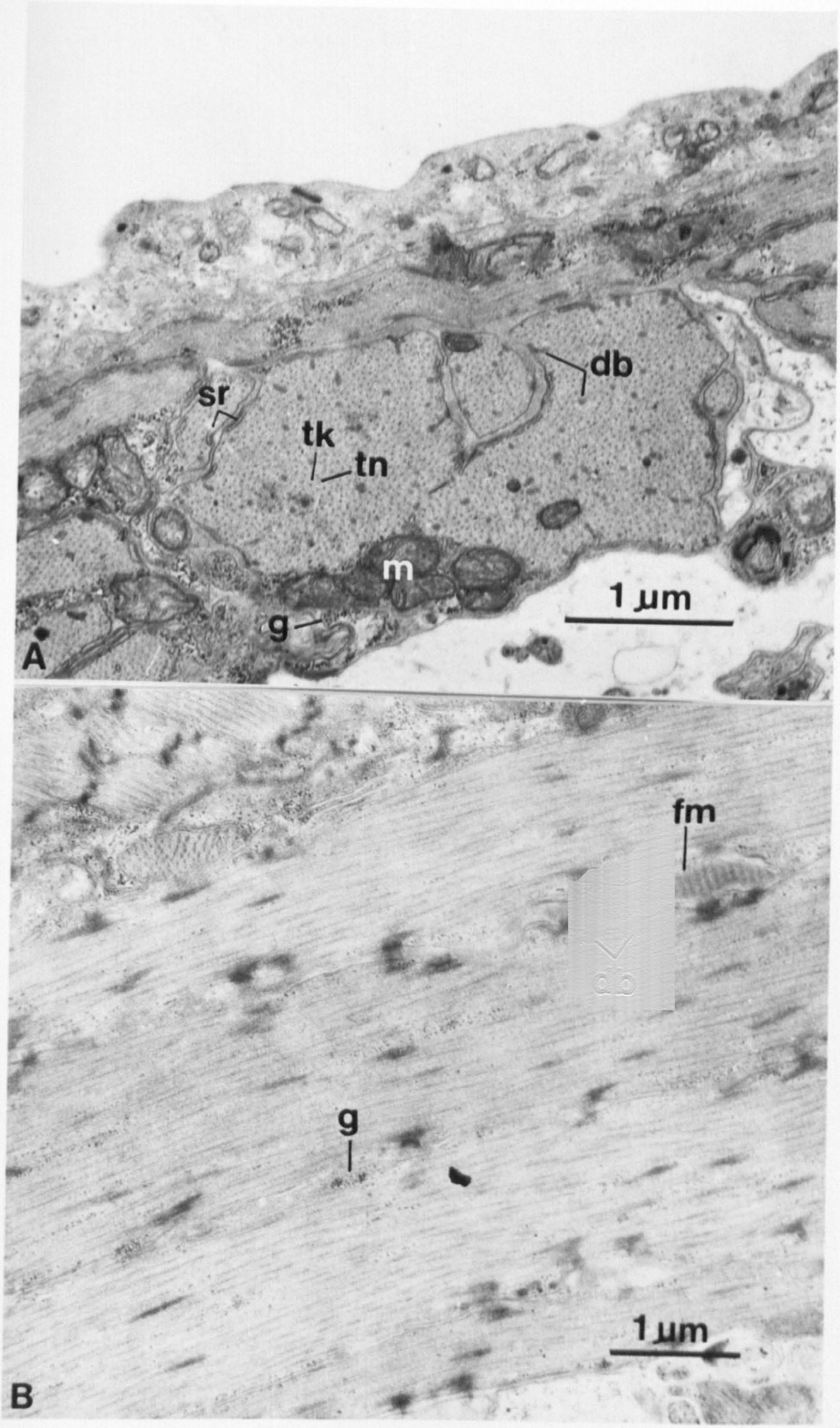

db
sr
tk
tn
m
g
1 µm
A
fm
g
1 µm
B

Plate 7.4 Transmission electron micrographs of longitudinal sections through the striated muscle fibres in the tail stem of cercariae

(A) Longitudinal section in the YZ plane, showing alternate light I band (I) and a dark A band (A) formed by the presence of thick (tk) and thin (tn) myofilaments. Transversely oriented Z rods (zr) and Z tubules (zt) of sarcoplasmic reticulum alternate. Cisternae of sarcoplasmic reticulum (sr) are present subjacent to the sarcolemma. Mitochondria (m) are large and abundant and contain many cristae.

(B) Longitudinal section in the XZ plane fixed in 2% potassium permanganate.

All cellular inclusions are oxidized and extracted; only the membrane structures are conserved. Note the mitochondria (m), cisternae of sarcoplasmic reticulum (sr) and Z tubules (zt).

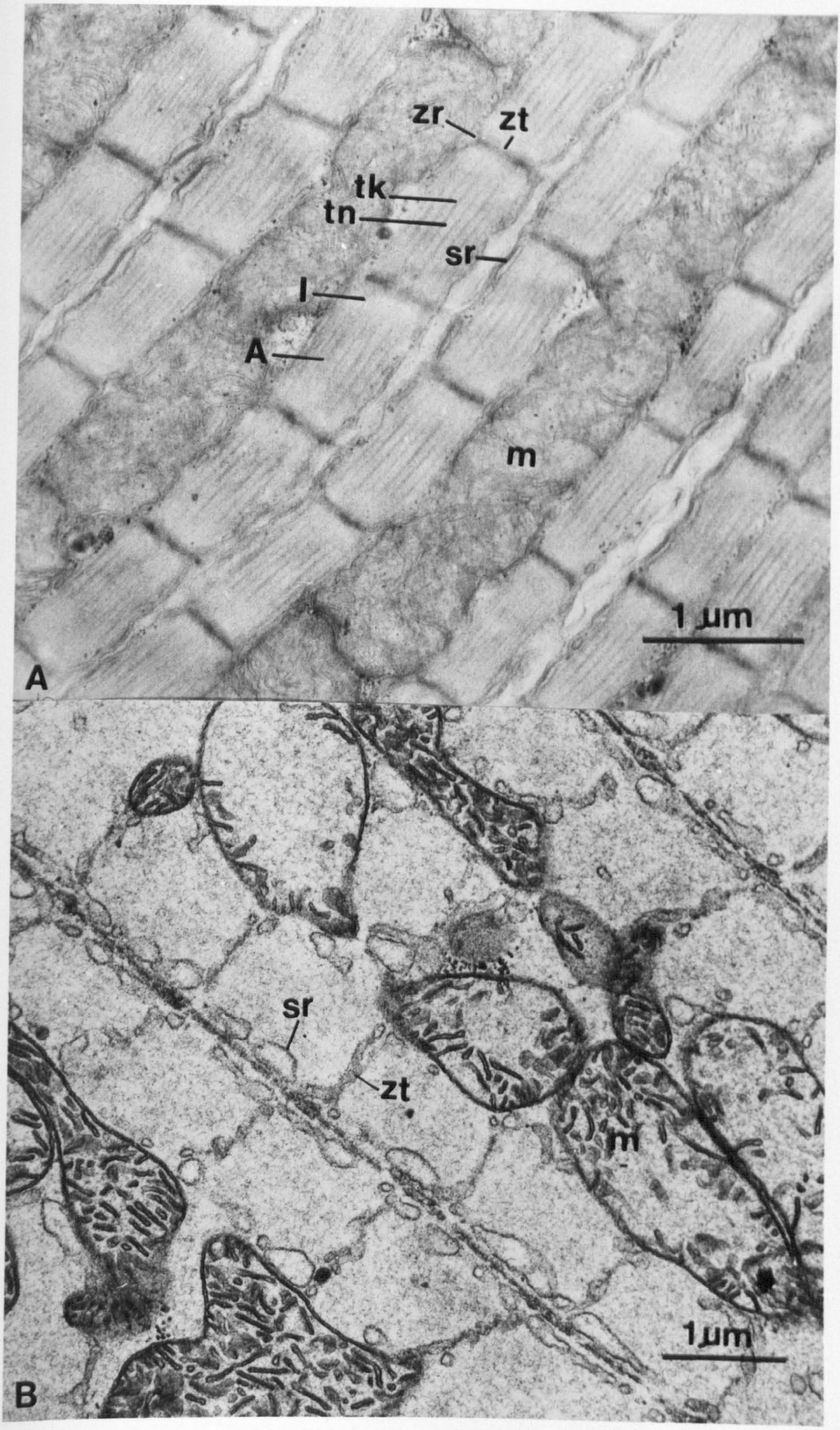

zr
zt
tk
tn
sr
I
A
m
1 µm
A
sr
zt
m
1µm
B

Plate 7.5 Transmission electron micrographs of transverse section in the XY plane of striated muscle fibres in the tail stem of cercariae.

(A) Two types of myofilaments, thick filaments (tk) and thin filaments (tn) are evident in the A band. Profiles of flattened cisternae of the sarcoplasmic reticulum (sr) are closely applied to the sarcolemma. Ovoid profiles of Z rods (zr) and Z tubules (zt) extend from the periphery of the contractile region to its central face.

(B) A section through an A band illustrating the characteristic arrangement of 10–12 thin myofilaments (tn) in a skewed hexagonal array around a single thick myofilament (tk).

(C) Z rods (zr) and Z tubules (zt) of the Z region have been cut longitudinally. Note the connection of Z tubules to subsarcolemmal cisternae.

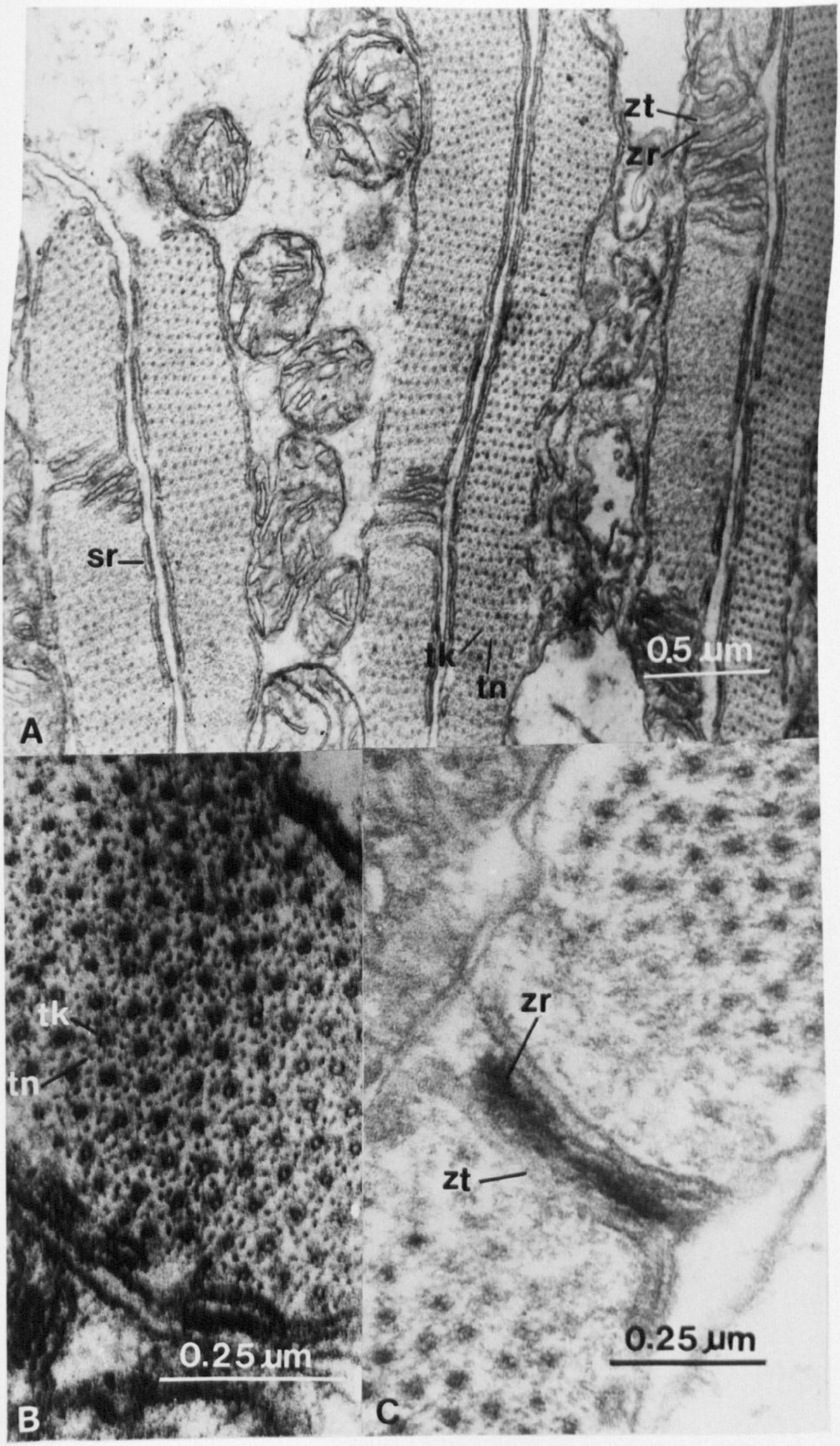

zt
zr
sr
tk
tn
0.5 µm
A
tk
tn
zr
zt
0.25 µm
B
0.25 µm
C

Plate 7.6 Transmission electron micrographs of longitudinal section in the XZ plane through the striated muscle fibres in the tail stem of cercariae

(A) The A band (A) and I band (I) as well as the H zone (H) are clearly defined. The thick (tk) and thin (tn) myofilaments lie approximately parallel to the longitudinal fibre axis. The Z region in the centre of the I band consists of alternating electron dense rods (zr) and tubules (zt). Large mitochondria (m) are present.

(B) Section through the Z region showing the Z rods (zr) and Z tubules (zt). Z rods are made up of a morphous dense material with laterally striated dense threads. Note thin myofilaments (tn) continuous with the cross sectioned profiles of the Z rods.

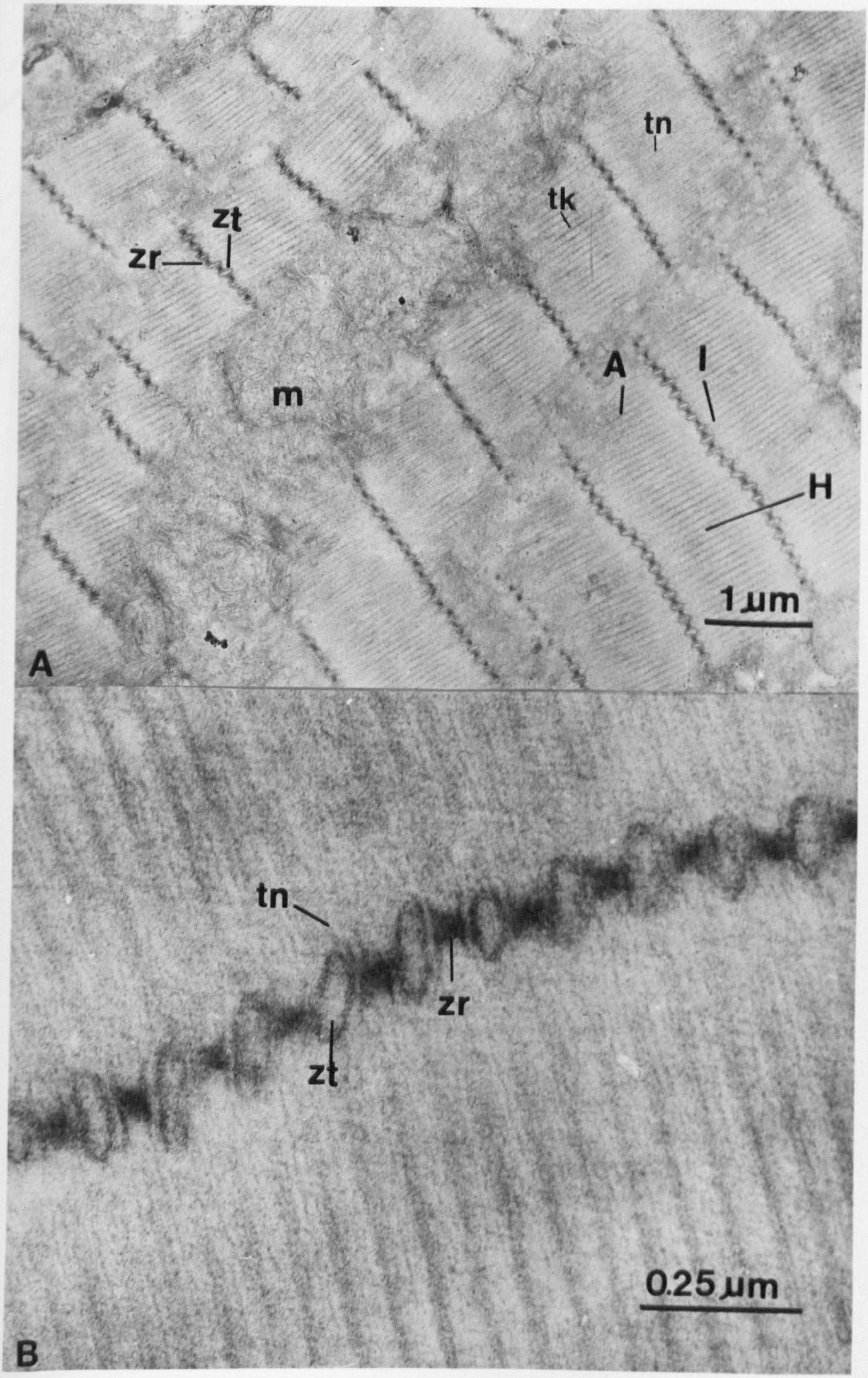

tn
tk
zt
zr
m
A
I
H
1 µm
A
tn
zr
zt
0.25 µm
B

Plate 7.7 Transmission electron micrograph of longitudinal section in the XZ plane through the striated muscle fibres

Longitudinal tubules of the sarcoplasmic reticulum (lt) enter between the myofilaments and into the Z line. The thick (tk) and thin (tn) myofilaments are clearly defined.

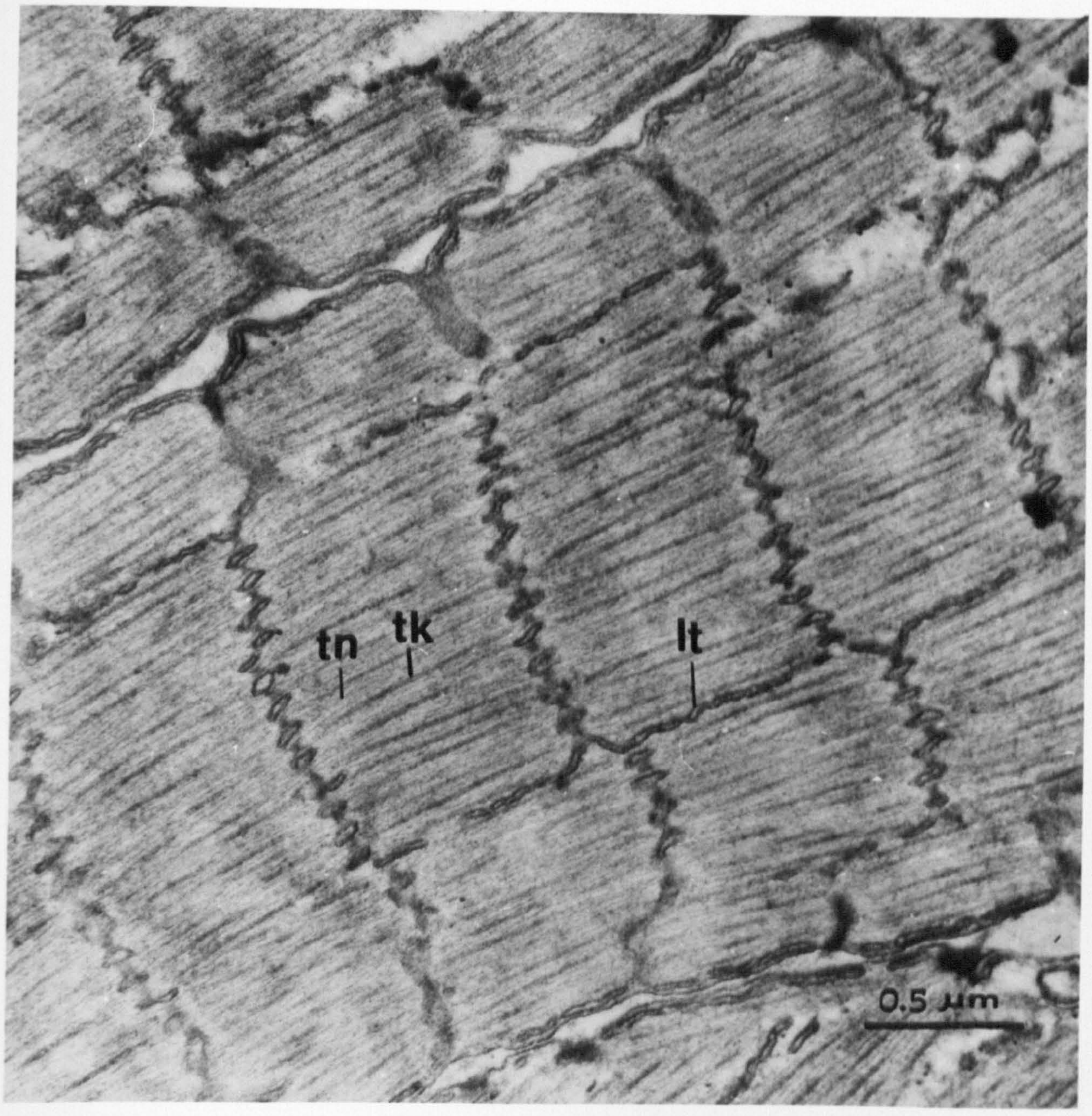

tn
tk
lt
0.5 µm

Plate 7.8 Transmission electron micrograph of a furca showing nucleus of longitudinal striated muscle fibre.

ch: peripheral chromatin clump
nlo: nucleolus

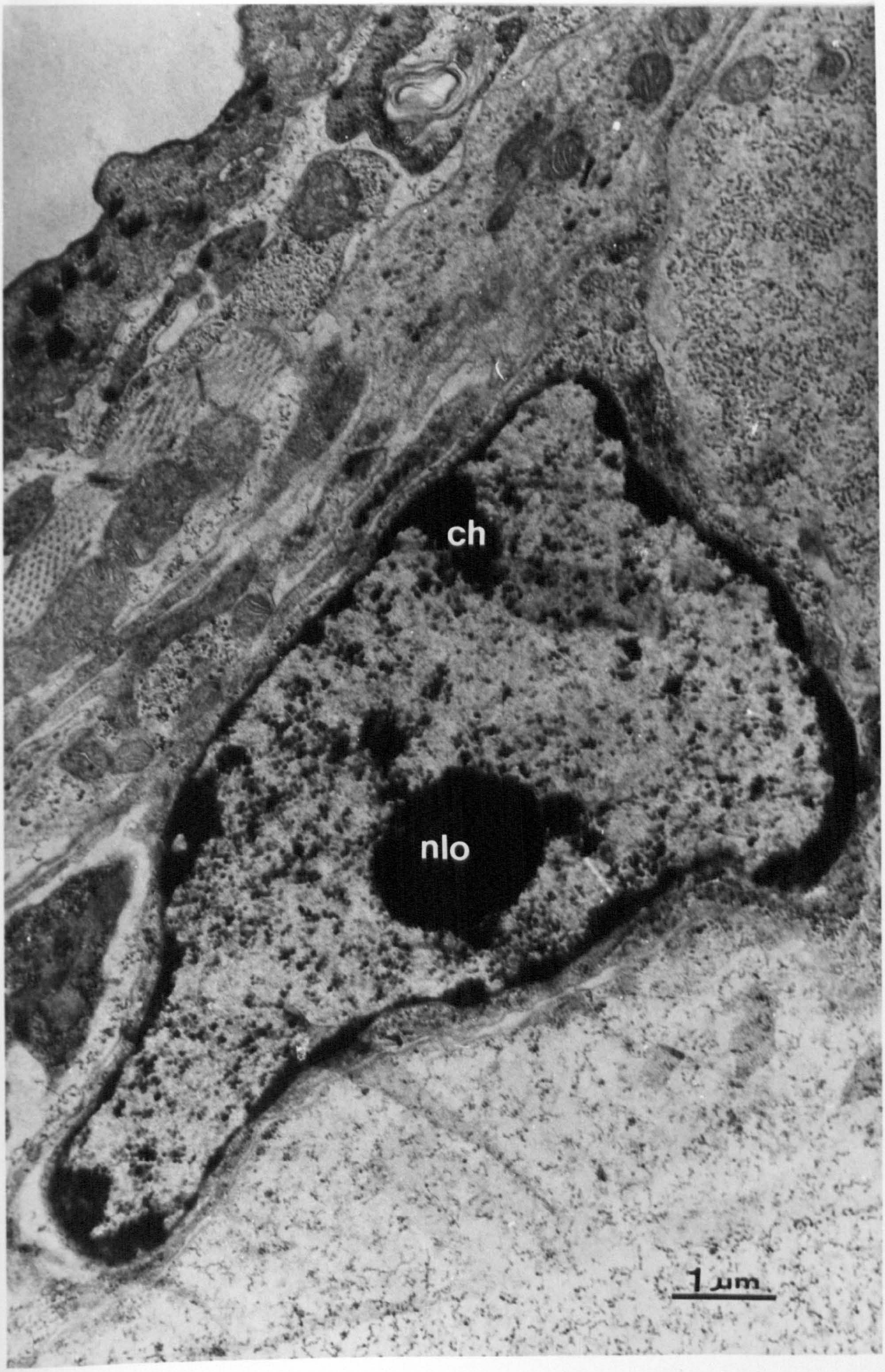

ch
nlo
1 µm

Plate 7.9 Transmission electron micrograph of a portion of a transverse section of the anterior region of the tail stem showing the appearance of the striated muscle fibres, proximal nerve mass and dorso-ventral non-striated muscle.

Key:

ax: axons of proximal nerve mass

bl: basal lamina

cm: circular non-striated muscle fibres

dvm: dorso-ventral non-striated muscle fibres

edi: electron dense inclusions in the tegument

lm: longitudinal non-striated muscle fibres

m: striated muscle fibre (m configuration)

se: sarcoplasmic extension of striated muscle cell

t: surface tegument

u: striated muscle fibre (U configuration)

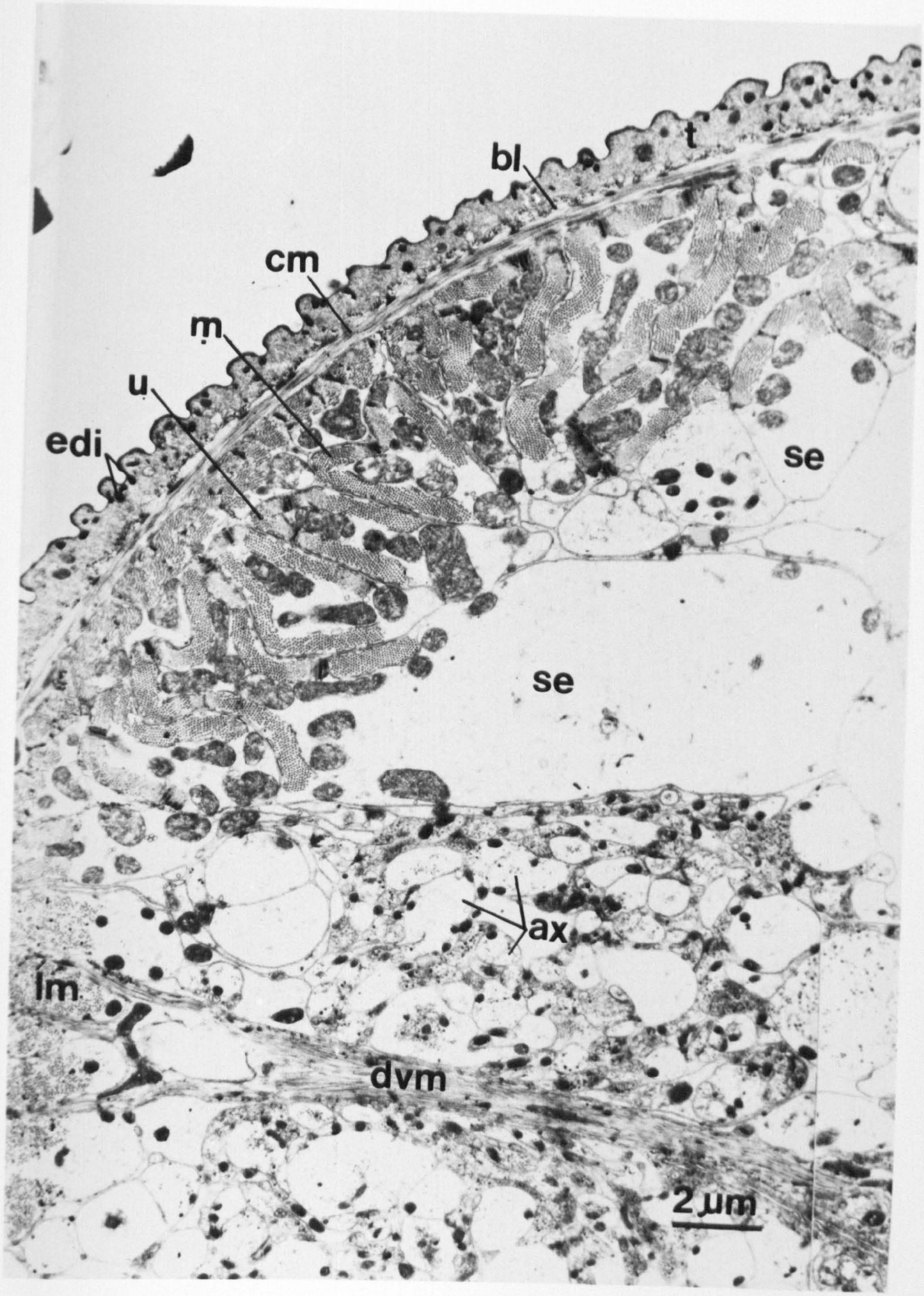

bl
t
cm
m
u
edi
se
se
ax
lm
dvm
2 µm

Plate 7.10 Transmission electron micrograph of a portion of a transverse section through the mid-region of the tail stem.

Circular non-striated muscle fibres (cm) and longitudinal muscle fibres (lm) lie beneath the surface tegument. Two blocks of striated muscle fibres (sm) appeared in this section. The myofibres are U shaped, producing a rosette pattern. Note the glycogen granules (g) and the dorsal nerve (dn).

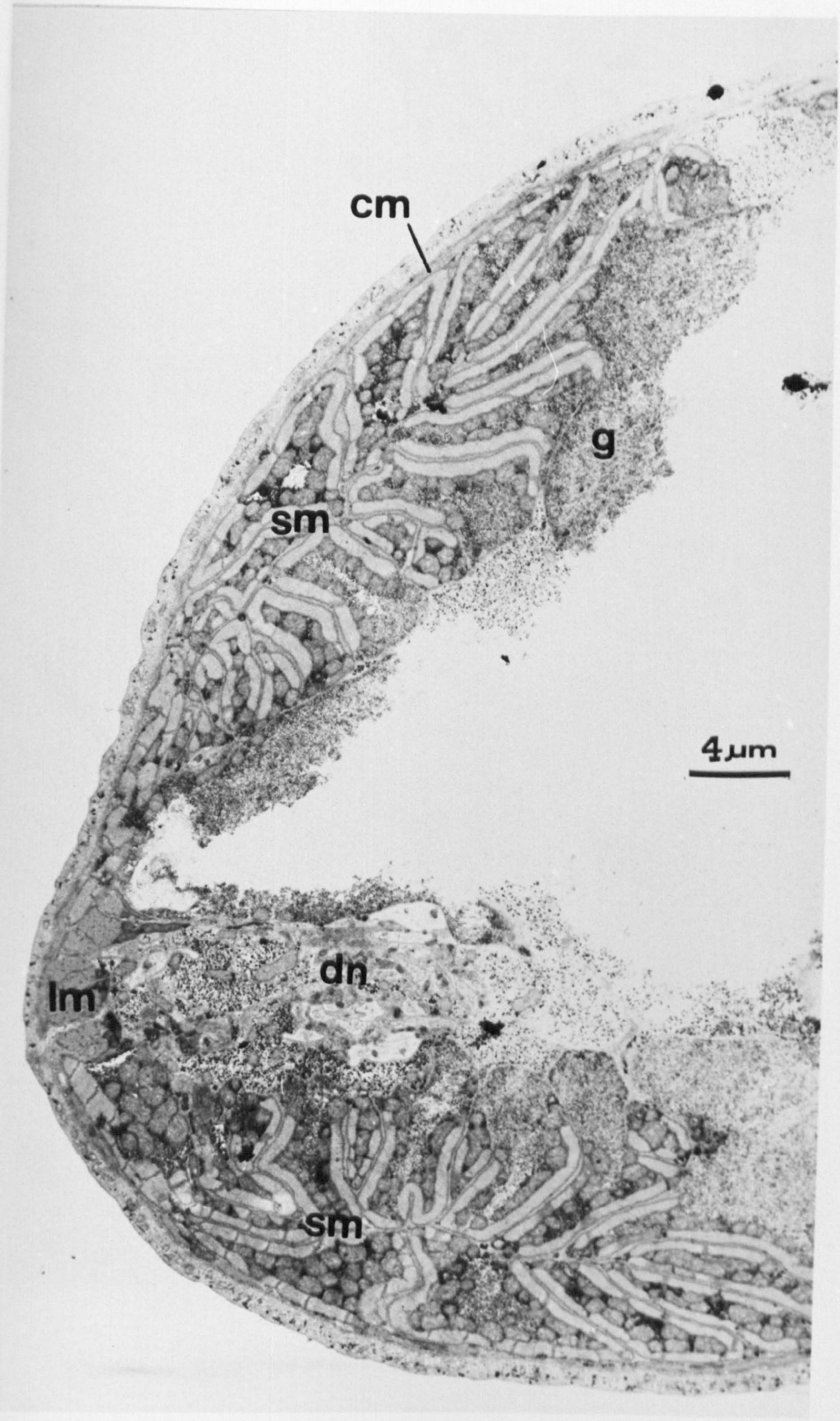

cm
g
sm
4 µm
lm
dn
sm

Plate 7.11 Transmission electron micrograph of a portion of a transverse section through the posterior region of the tail stem.

Circular non-striated muscle fibres (cm) and longitudinal non-striated muscle fibres (lm) lie beneath the tegument. The striated muscle fibres exhibit several configurations. A single layer of contractile process profiles subtending the circular myofibre layer and exhibit both double m shaped profiles (mm) and solid ones (so). Dorsally or ventrally the layer of the contractile processes becomes double. The inner layer of contractile processes has U-shaped (U) or m shaped (m).
dnm: distal nerve mass.

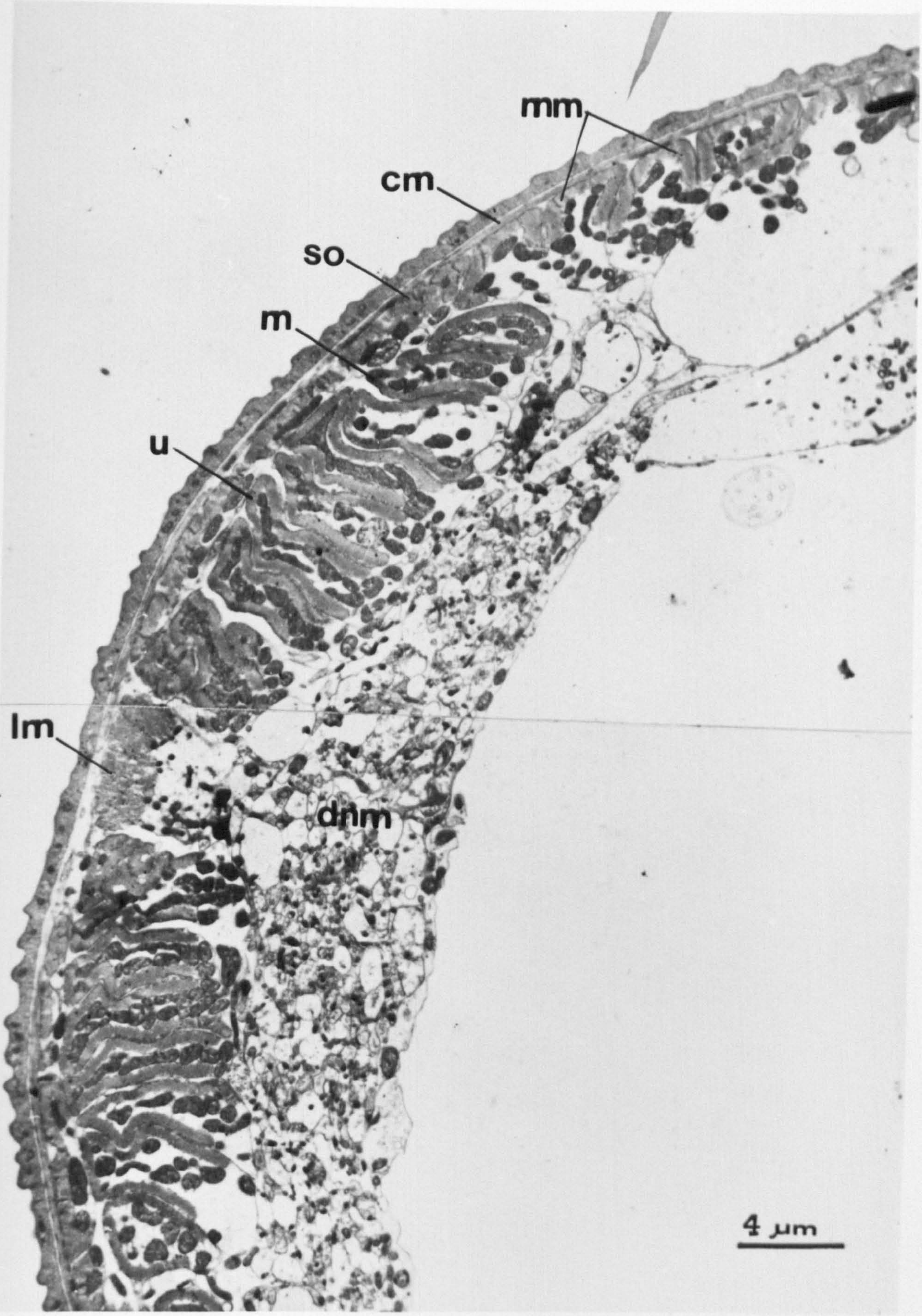

mm
cm
so
m
u
lm
dnm
4 µm

Plate 7.12 Transmission electron micrograph of the tail stem/furcal junction

The tegument (t) in this region is highly folded and parts of the cruciform muscle system (ccf) are visible

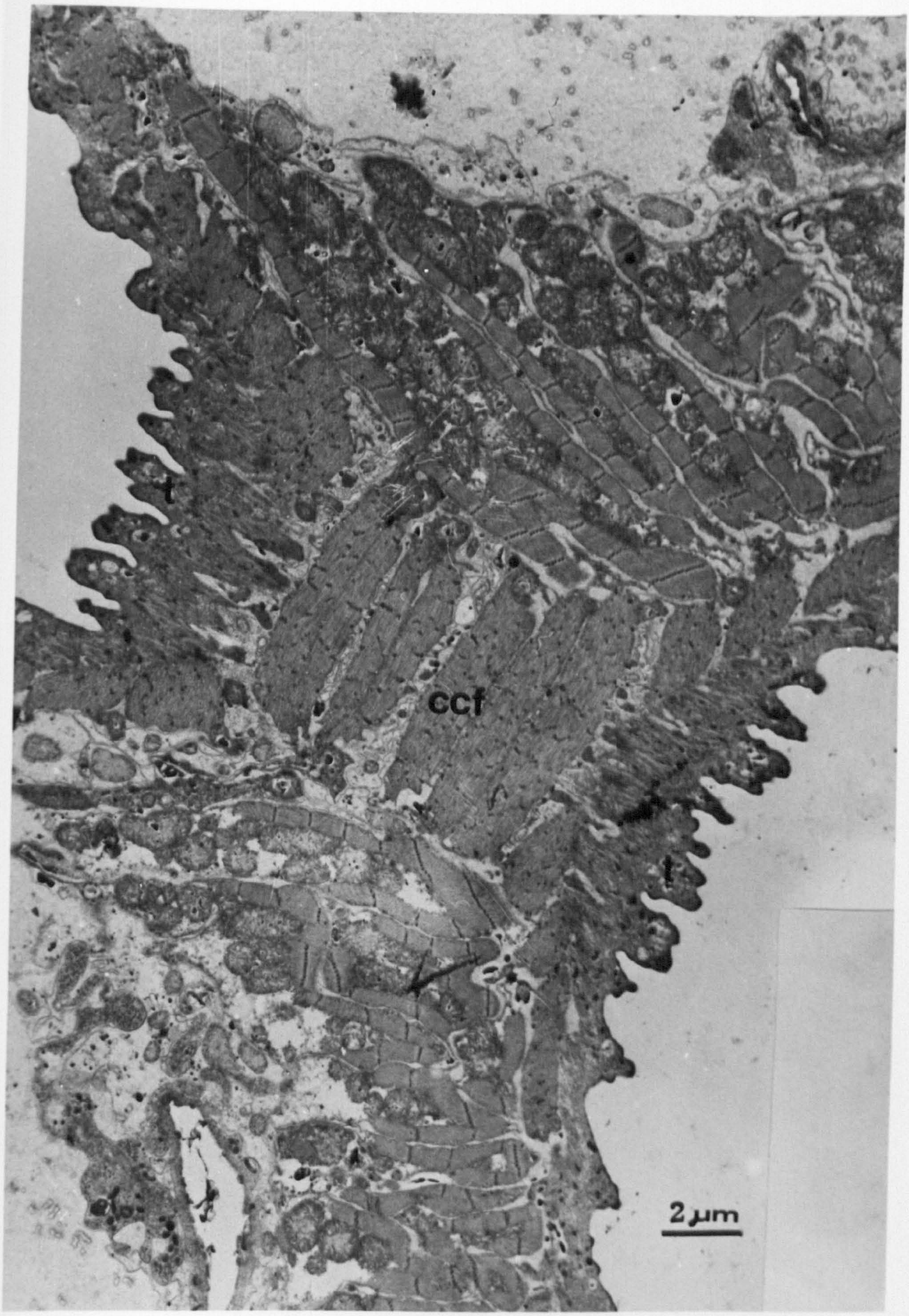

ccf
2 µm

Plate 7.13 Transmission electron micrograph of a transverse section through an arm process.

ag: adhesive granules in adhesive pad of arm process
cm: circular non-striated muscle fibre surrounds the arm core
lm: longitudinal non-striated muscle fibre in two clusters of fibres
sm: striated muscle fibres in two asymmetrical blocks

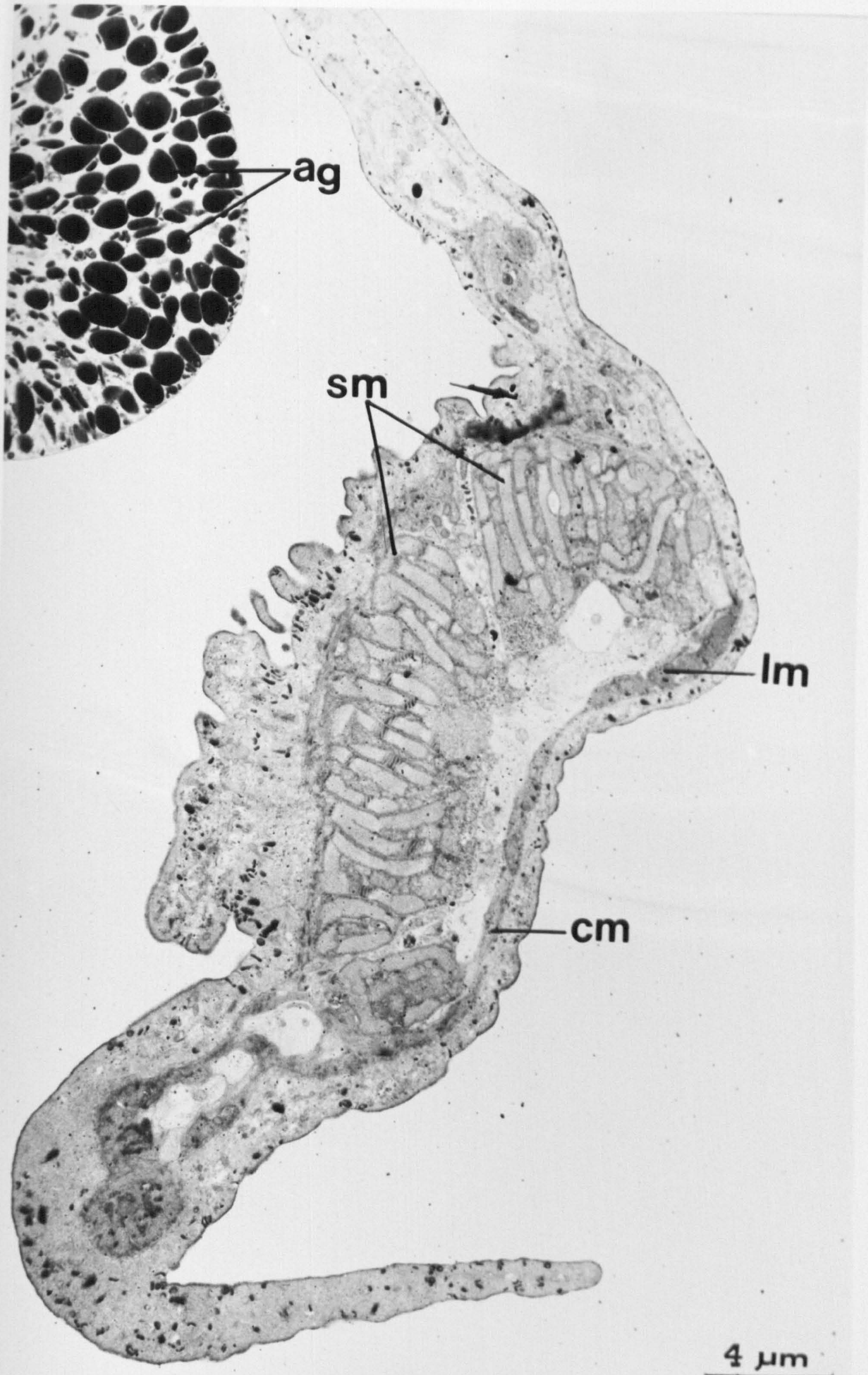

ag
sm
lm
cm
4 µm

Plate 7.14 Transmission electron micrograph of a cross section through a furca.

cm: circular non-striated muscle fibre beneath the basal lamina
lm: longitudinal non-striated muscle fibre restricted to the medial face
sm: striated muscle fibre arranged on the medial and lateral face of the furca

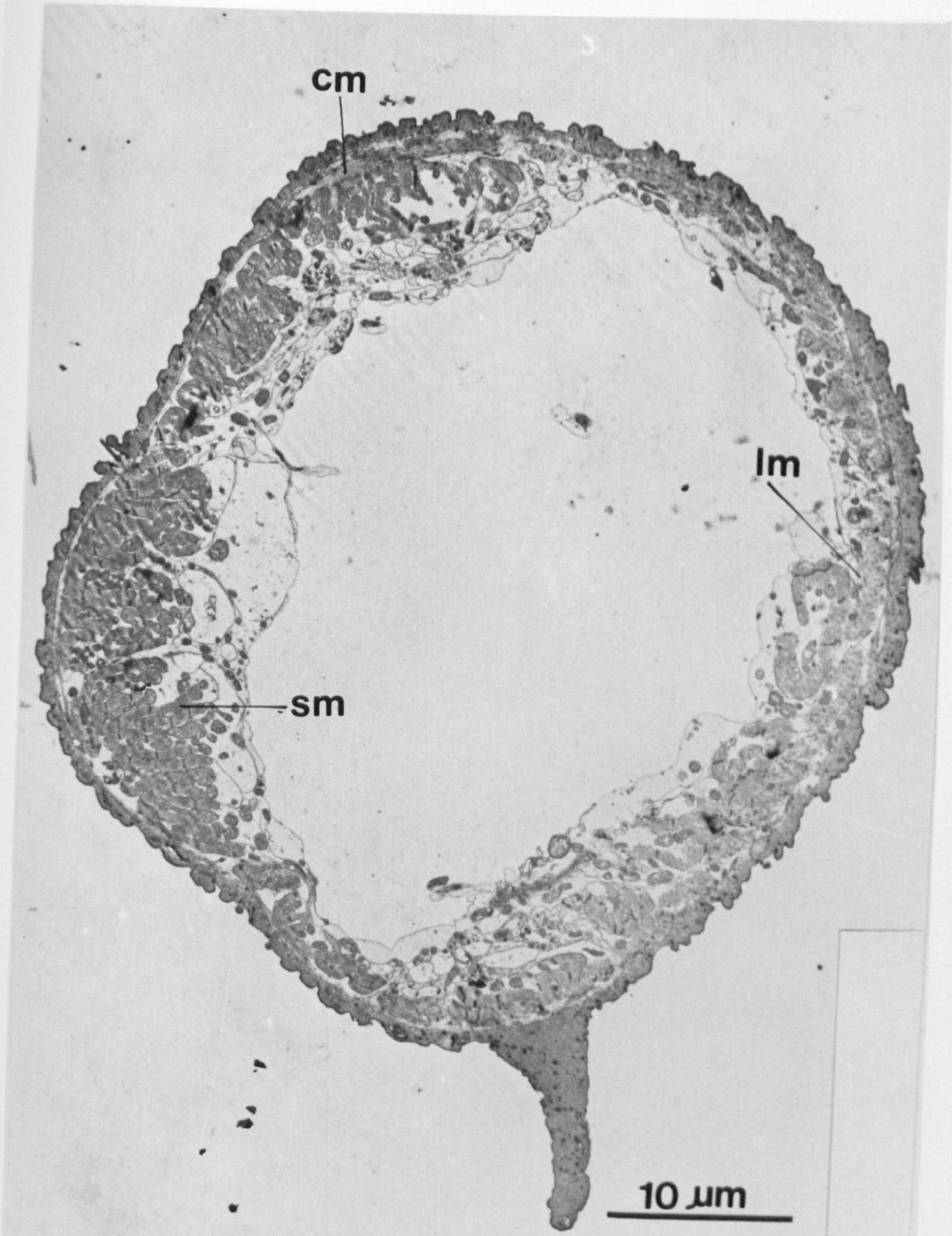

cm
lm
sm
10 µm

Plate 7.15 Transmission electron micrograph of a transverse section through a furca.

Circular non-striated muscle fibres (cm) and striated muscle fibres (sm). A nerve bulb (nb) in tegument possesses a sensory cilium.

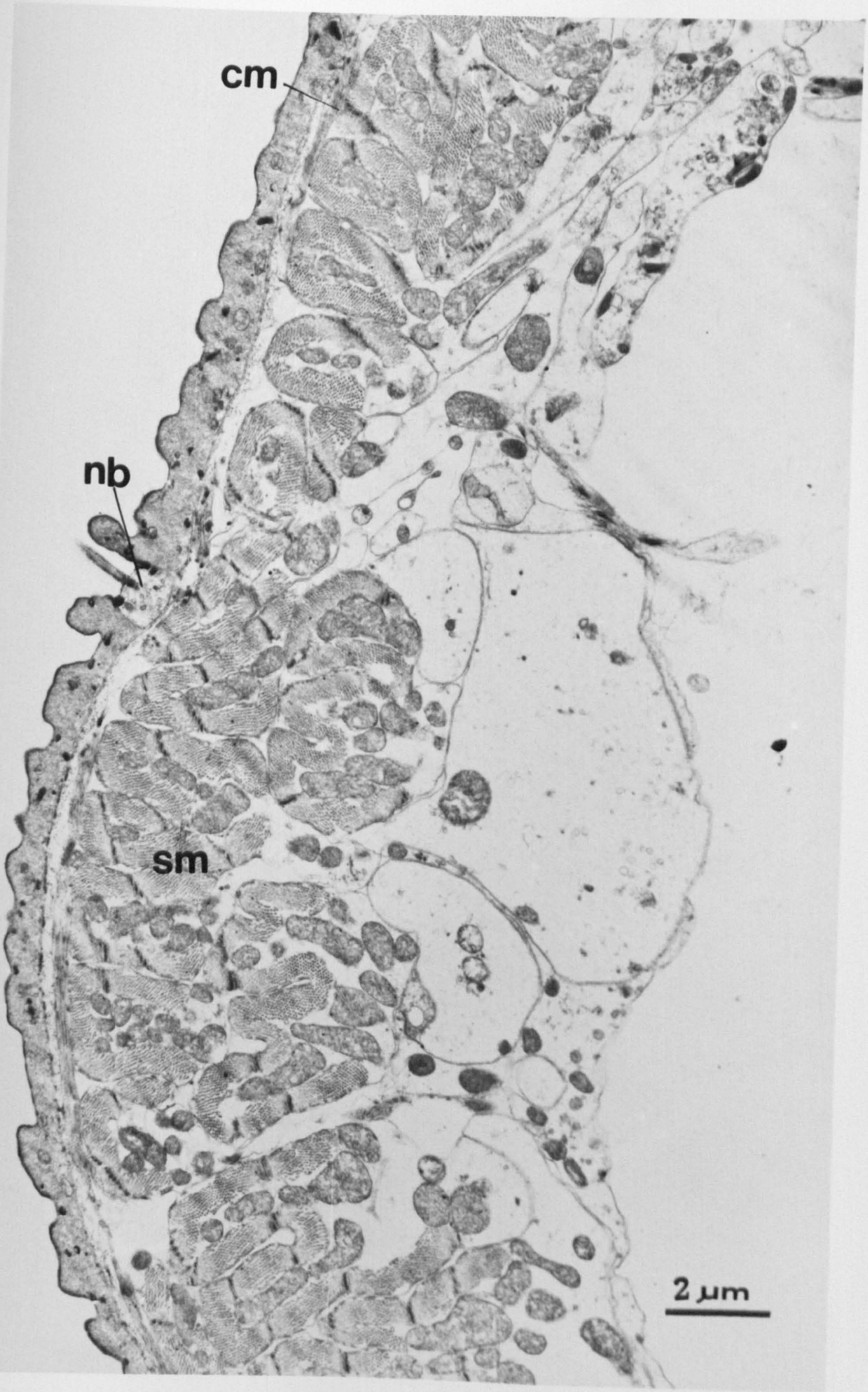

cm
nb
sm
2 µm

Plate 7.16 Transmission electron micrograph of horizontal section of furca showing the transverse non-striated muscle fibres (tm) inserted into the basal lamina.

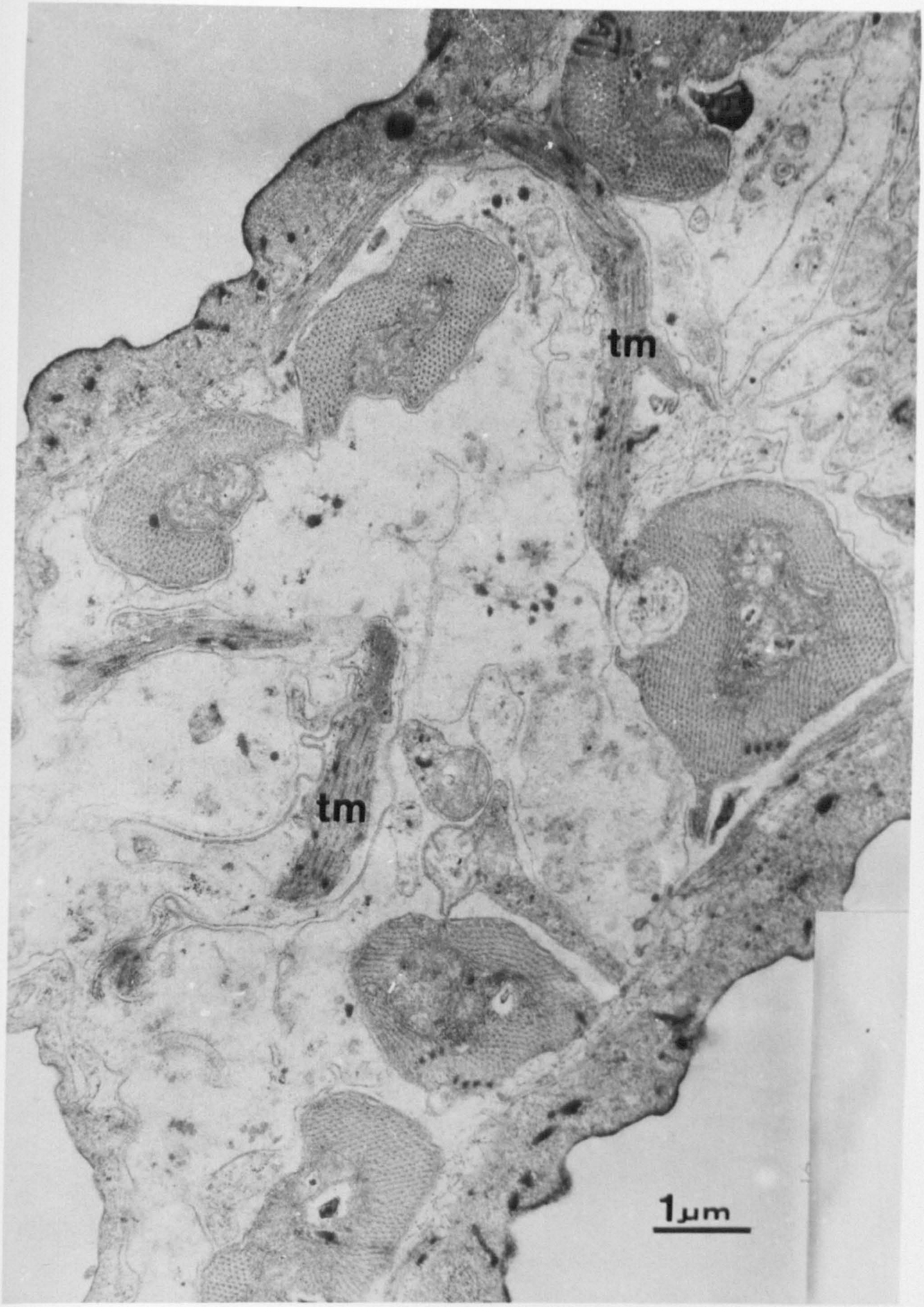

tm
tm
1µm

Plate 7.17 Transmission electron micrographs of the tail/head junction (horizontal section).

(A) blm: longitudinal non-striated muscle in the body wall of the head
jz: junctional zone
sm: striated muscle of the tail terminates behind the junctional zone
t: folded tegument
tlm: tail longitudinal non-striated muscle fibre

(B) Part of excretory bladder (ex) closely adjacent to the region of junction between head (h) and tail (t); nerve axons (ax) pass between head and tail.

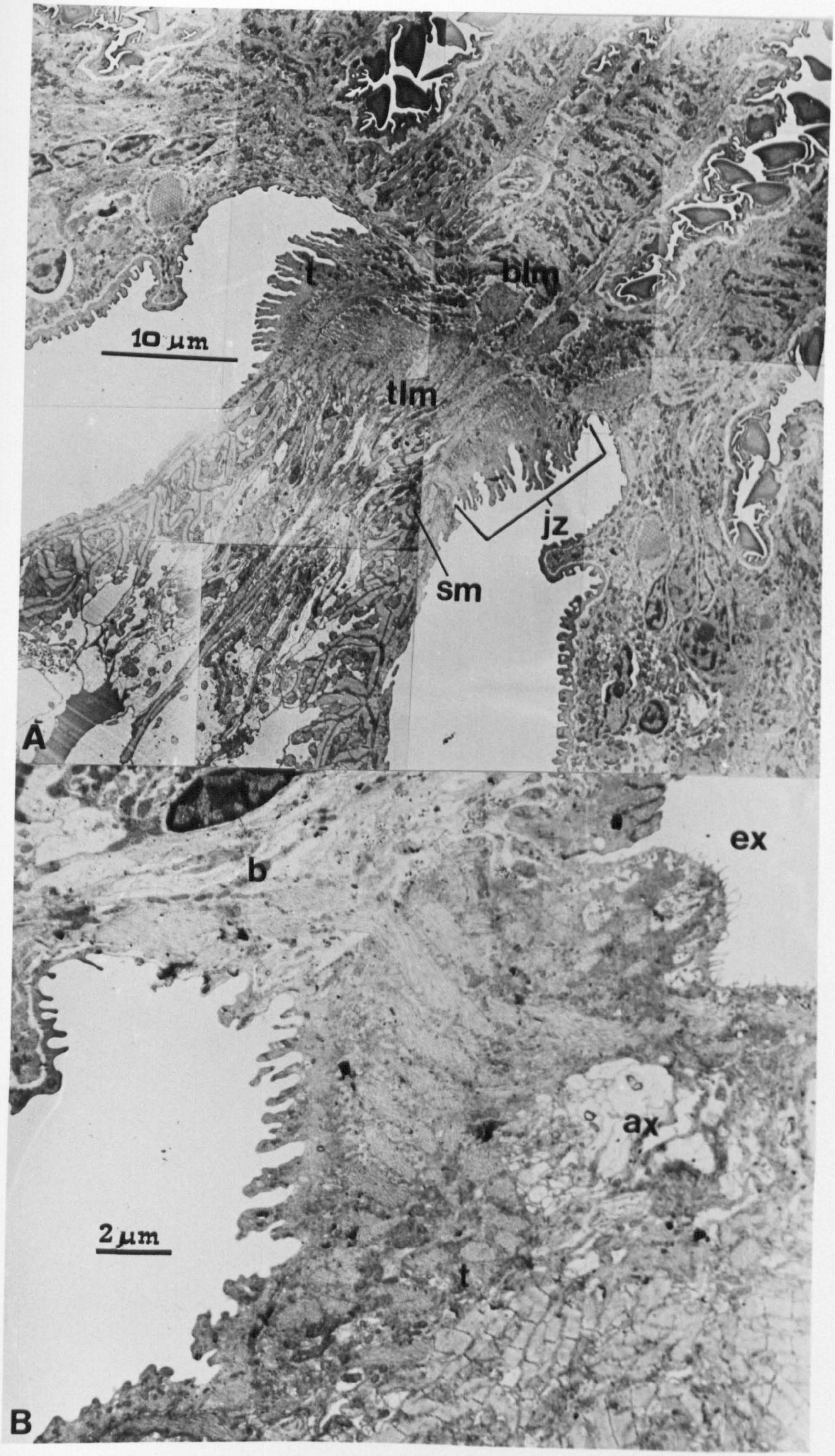

10 µm
t
blm
tlm
jz
sm
A
b
ex
ax
2 µm
t
B

Plate 8.1 Transmission electron micrograph of a region of cercarial cerebral ganglia.

The ganglion consists of neuropile (nu) with unmyelinated nerve fibres and a peripheral layer containing three types of nerve cell bodies.
1. Type 1A has a large nucleus (n) with a nucleolus and a prominent chromatin network. The perinuclear cytoplasm contains a high density of free ribosomes (r), few mitochondria (m) and small amount of granular endoplasmic reticulum (er).
2. Type 1B cells contain medium diameter dense_cored inclusions (mdi).
3. Type 2 perinuclear cytoplasm contains prominent cisternae of endoplasmic reticulum (er) and a small number of large diameter inclusions (ldi).

Small non-striated muscle fibres (lm) are associated with the neuropile and peripheral layer.

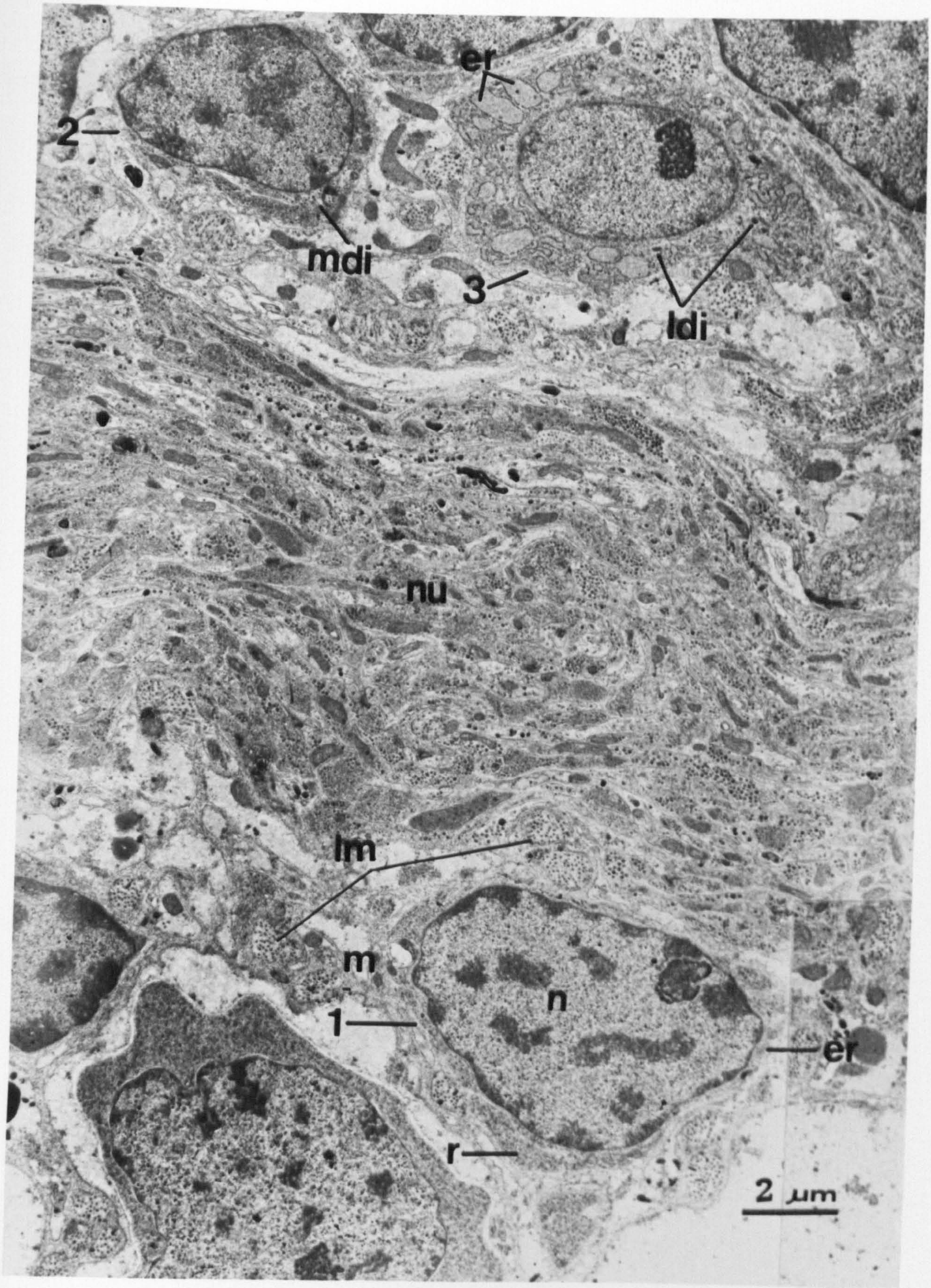

er
2
mdi
3
ldi
nu
lm
m
n
1
er
r
2 µm

Plate 8.2 (A) Transmission electron micrograph of the proximal caudal nerve mass

ax: axon profiles

cv: small clear vesicles

m: mitochondrion

mdi: medium diameter dense-cored inclusion

(B) Transmission electron micrograph of the distal caudal nerve mass

ax: axon profiles

cv: small clear vesicles

m: mitochondrion

mdi: medium diameter dense-cored inclusion

sm: striated muscle fibre

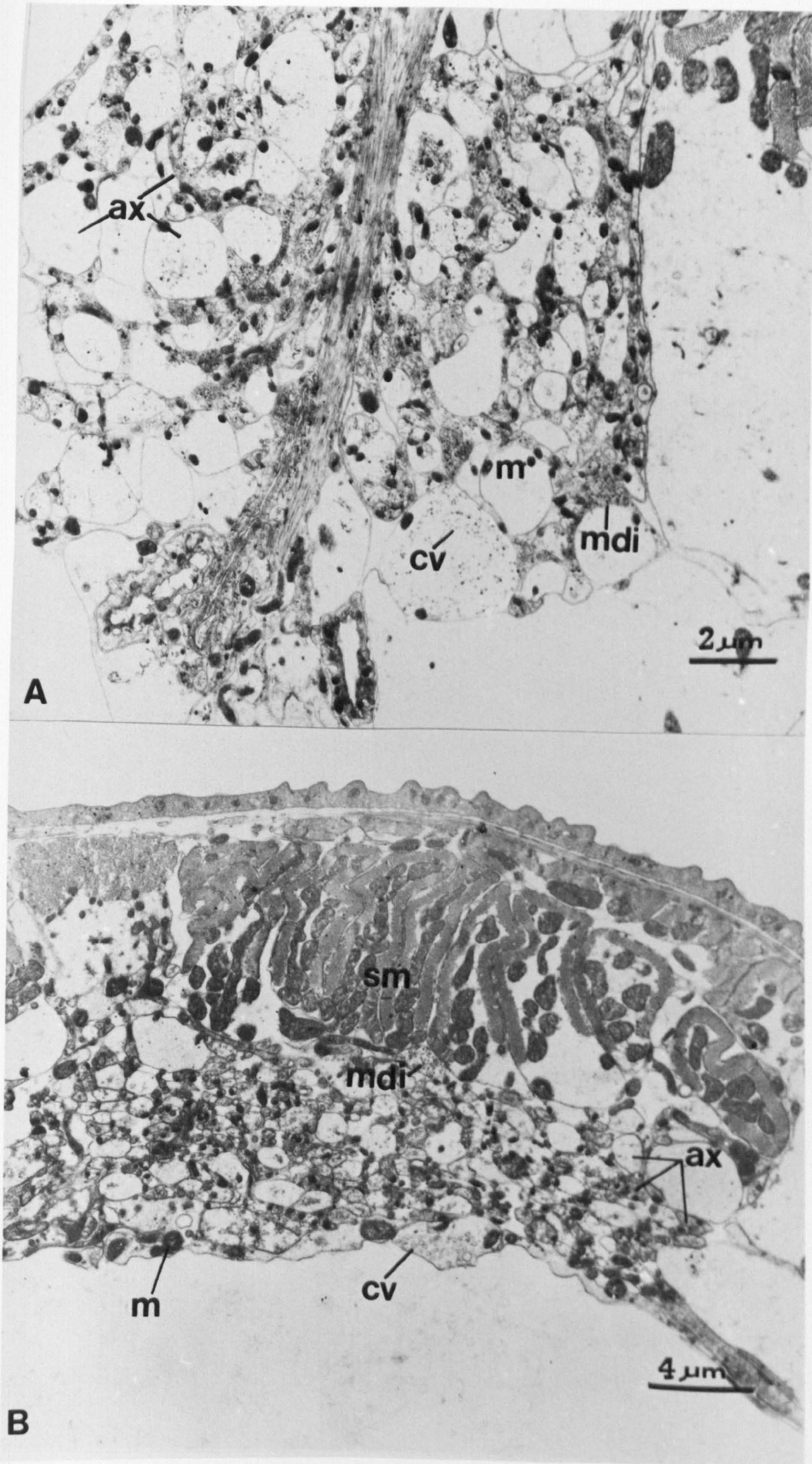

ax
m
cv
mdi
2 µm
A
sm
mdi
ax
m
cv
4 µm
B

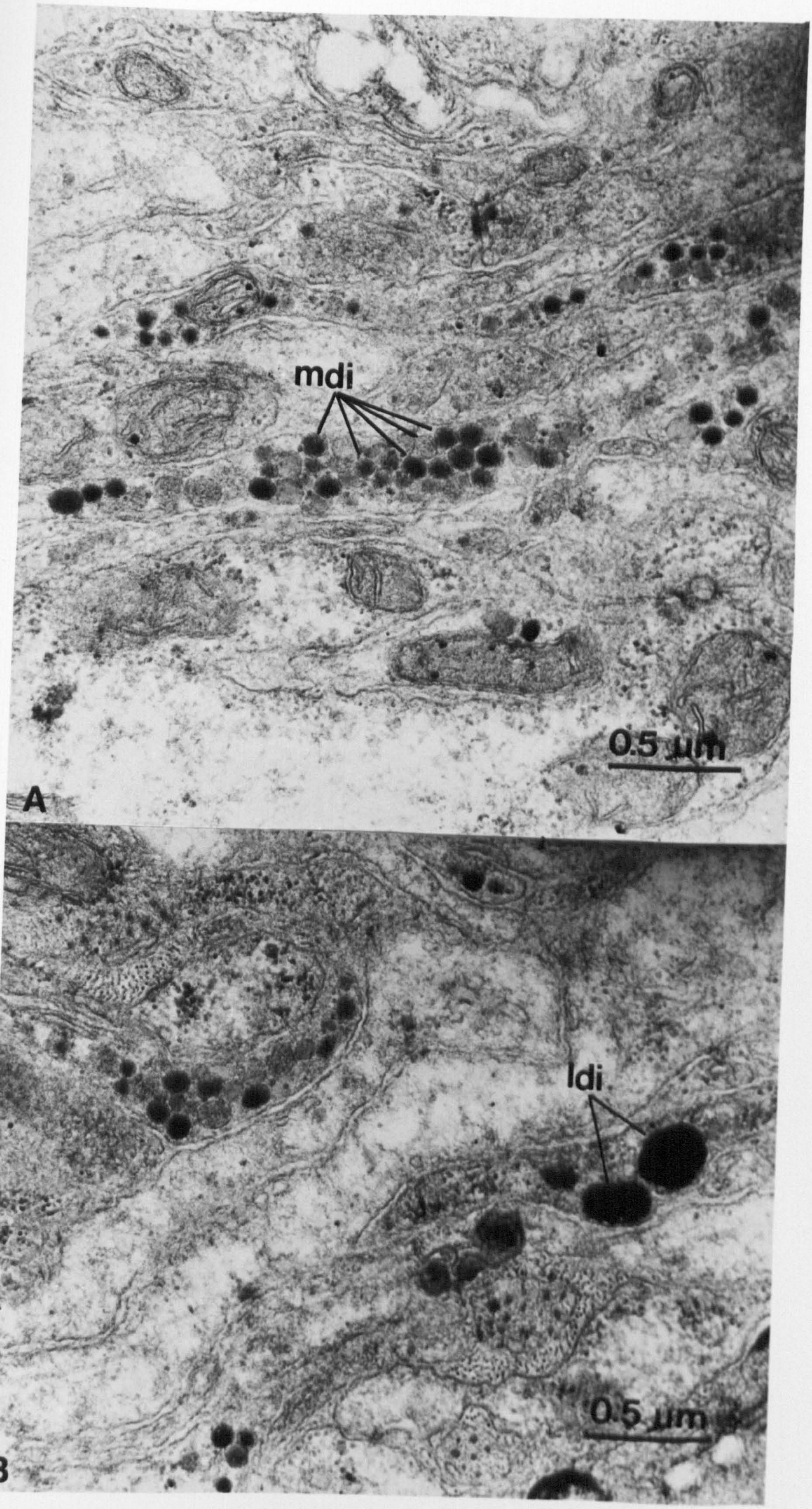
Plate 8.3 (A) Transmission electron micrograph of dorsal longitudinal nerve cord.
lax: large diameter axon
m: mitochondrion
nt: neurotubule
sax: small diameter axon

(B) Transmission electron micrograph of the neuropile of the cerebral ganglia illustrating various sizes of unmylinated axonal and dendritic processes.
cv: small clear vesicle
lm: cross section of longitudinal non-striated muscle fibre
m: mitochondrion
mdi: medium diameter dense-cored inclusions
nt: neurotubule


sax
nt
m
lax
4 µm
A
m
lm
mdi
cv
nt
1 µm
B

Plate 8.4 (A) Transmission electron micrograph of cerebral ganglion neuropile illustrating the range of appearance of medium diameter dense-cored inclusions (mdi).

(B) Transmission electron micrograph of cerebral ganglia neuropile with a dendrite containing large diameter dense inclusions (ldi).


mdi
0.5 µm
A
ldi
0.5 µm
B

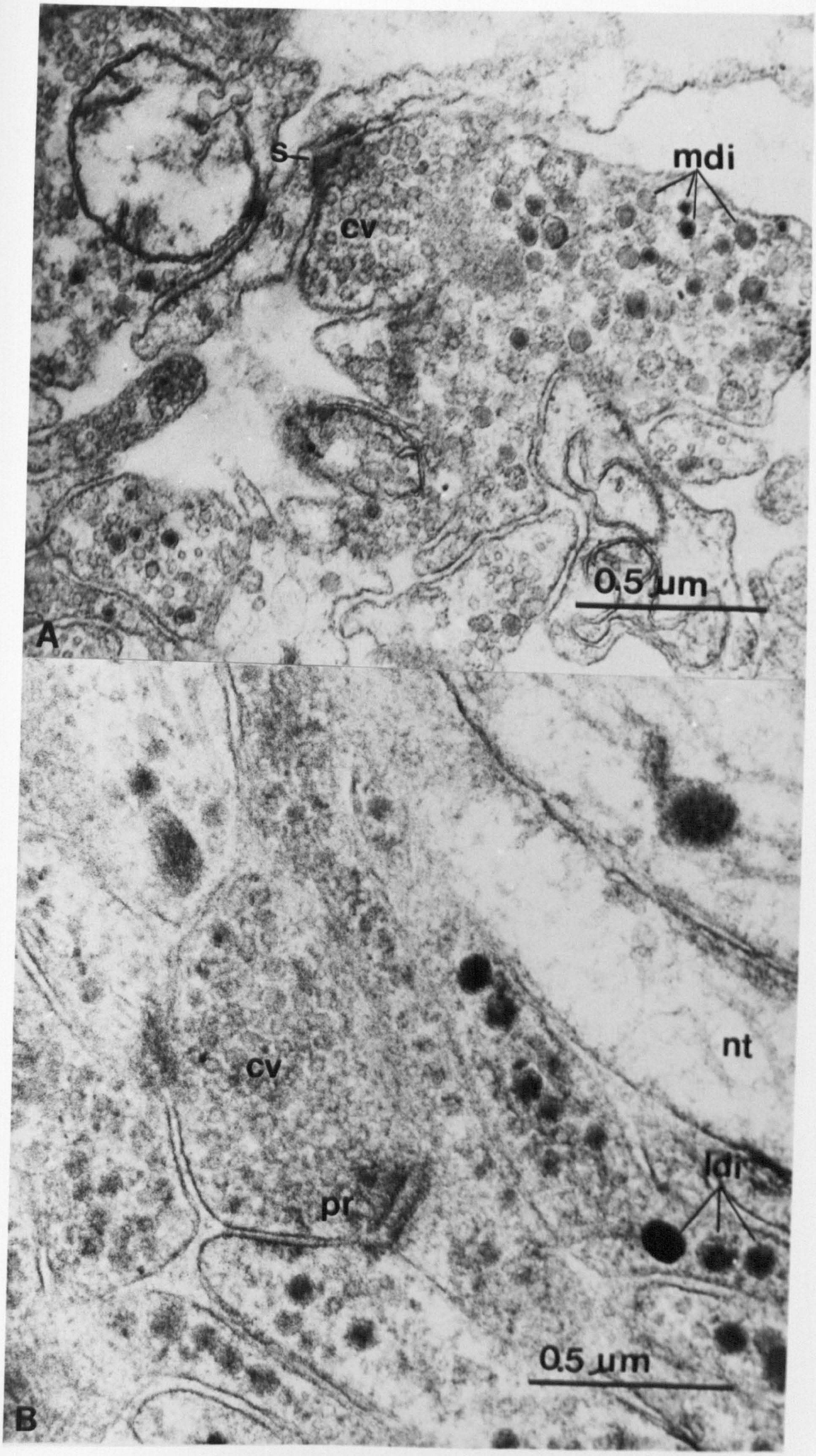

s
cv
mdi
0.5 µm
A
cv
pr
nt
ldr
0.5 µm
B

Plate 8.6 (A) Transmission electron micrograph of a synaptic junction (nerve-nerve)in a caudal longitudinal nerve. Note the thickened post-synaptic membrane and a synaptic cleft filled with dense material (cd).

(B) Transmission electron micrograph of a simple synapse (s) in the nerve fibres of the tail with accumulation of small clear vesicles (cv) in the pre-synaptic terminal.

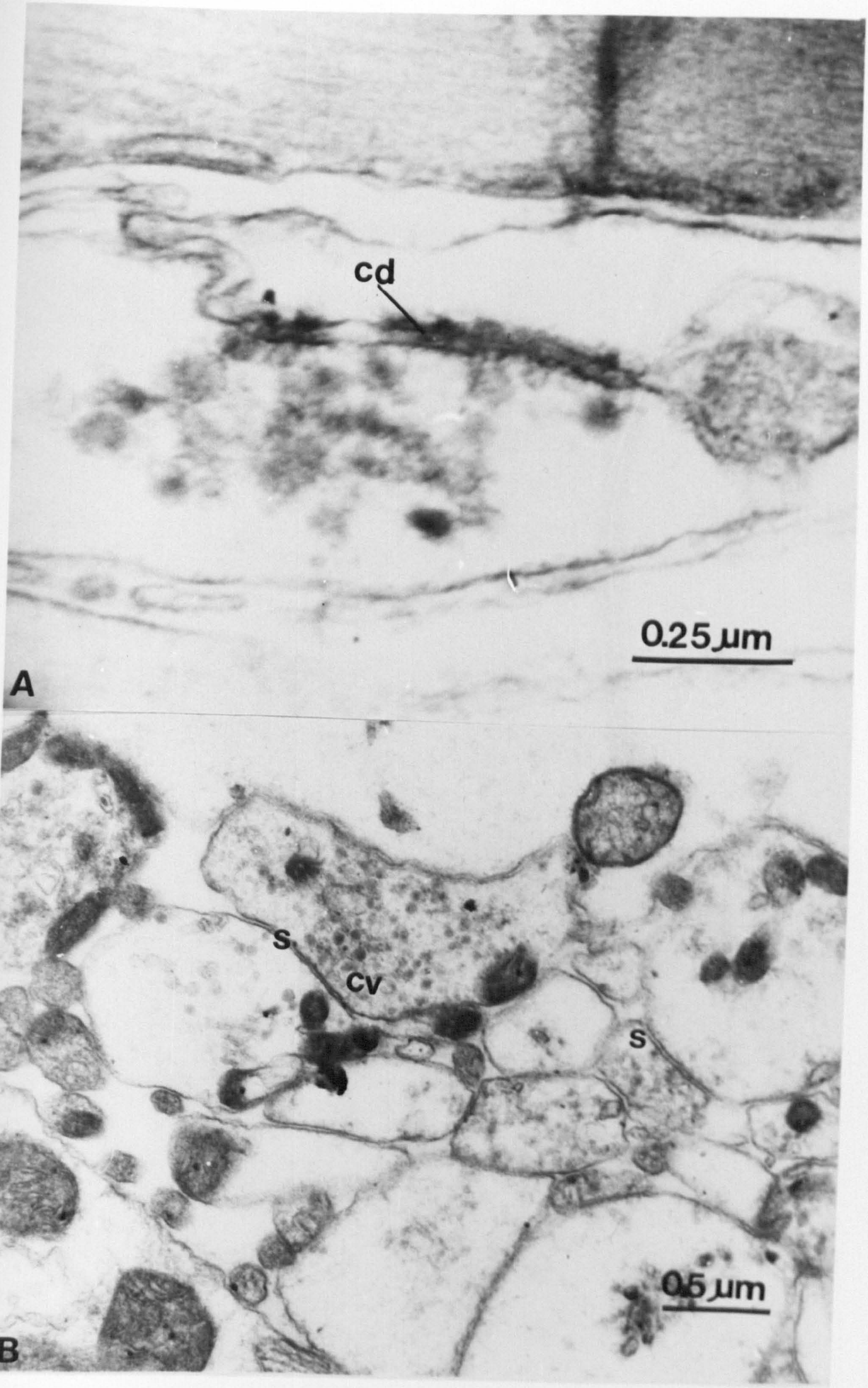

cd
0.25 µm
A
S
CV
S
0.5 µm
B

Plate 8.7 Transmission electron micrograph of a caudal neuromuscular junction.
A nerve ending contact with a caudal non-striated muscle fibre (cm). No specialised pre- and post-synaptic structures are seen. The dendrite contains a mixture of a small clear vesicle (cv), medium diameter dense-cored inclusions (mdi), mitochondria (m) and neurotubules (nt).

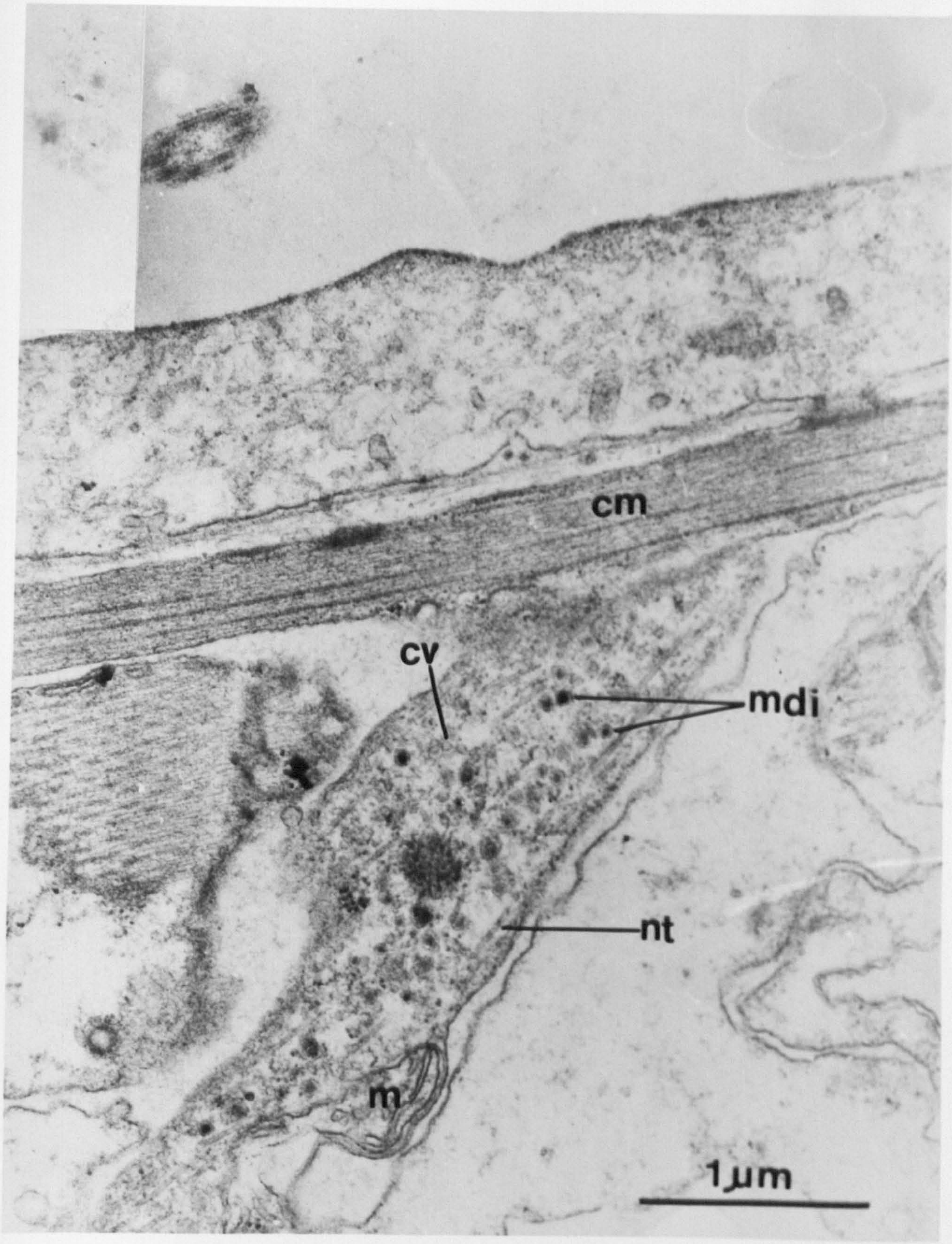

cm
cv
mdi
nt
m
1µm

Plate 8.8 Transmission electron micrograph of a caudal neuromuscular junction

A type 1 neuromuscular junction (nerve-striated muscle cell) with simple cleft (c).
The post-junctional membrane (po) is thickened with tuft-like dense projections. Small clear vesicles (cv) are obvious in the pre-synaptic terminal.

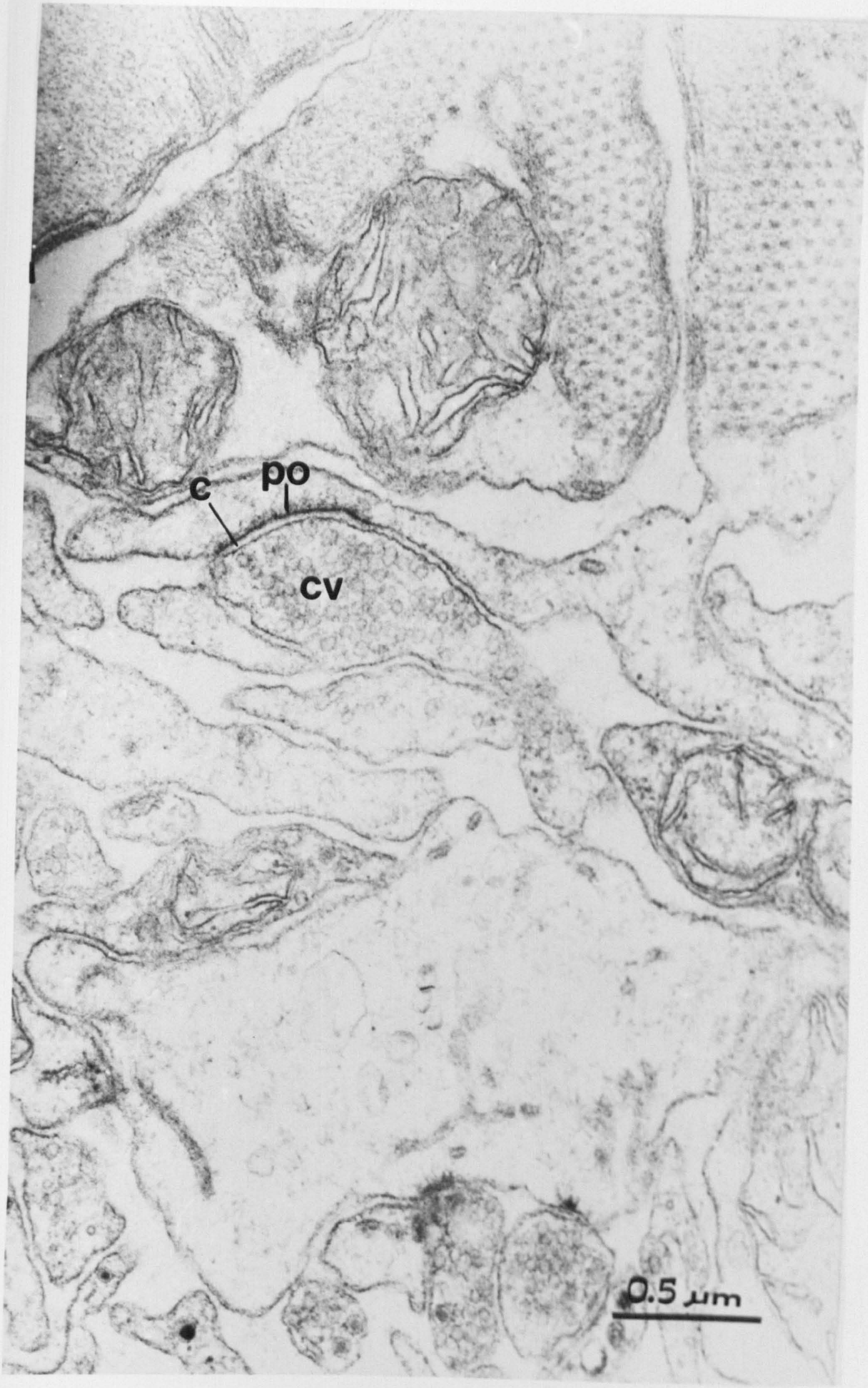

c
po
cv
0.5 µm

Plate 8.9 Transmission electron micrographs of caudal neuromuscular junctions

(A) A type 2 (1) and a type 4 (2) neuromuscular junction on the same sarcoplasmic process of a striated muscle fibre.

(B) A type 4 neuromuscular junction.
The junctional cleft has a central band of dense material (cd) and the post-junctional membrane thickened with tuft-like projections (po). The pre-synaptic terminal contains a cluster of medium-diameter dense cored inclusions (mdi) together with some small clear vesicles (cv)

(C) A type 2 neuromuscular junction.
With filled cleft and the post junctional membrane thickened with tuft-like dense projections (po). Note the pre-synaptic density (prd) and the cluster of small clear vesicles (cv)

(1)

A

(2)

0.5 µm

mdi

cd

po

cv

B

0.5 µm

prd

cv

po

C

0.5 µm

Plate 8.10 Transmission electron micrograph of a caudal neuromuscular junction

A type 3 neuromuscular junction with fine dense material (td) in the junctional cleft running parallel to the pre- and post-synaptic membrane. The post-junctional membrane is thickened with tuft-like projections (po) and small clear vesicles (cv) are present in the pre-synaptic terminal

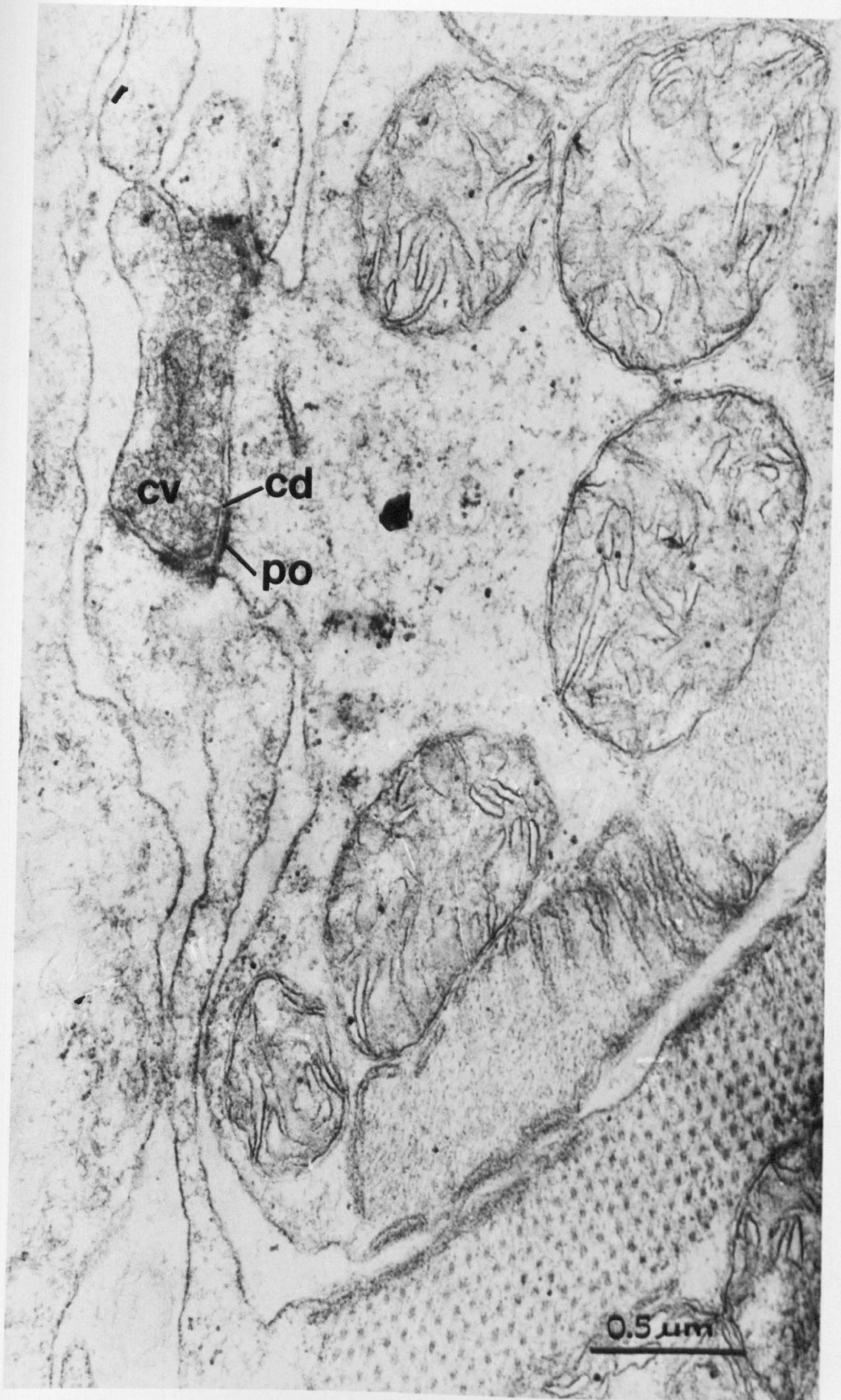

cv
cd
po
0.5 µm

Plate 8.11 Transmission electron micrograph of a cluster of caudal neuromuscular junctions

A single nerve ending is in intimate contact with three separate striated muscle processes

Type 5 neuromuscular junction (arrow) showing simple cleft (c), post-junctional membrane revealing electron density with tuft-like projection (po). The pre-synaptic terminal packed with medium diameter dense-cored inclusions (mdi) and a few small clear vesicles (cv). The other two processes (a and b) are in close contact with the nerve terminal with no membrane specialisations

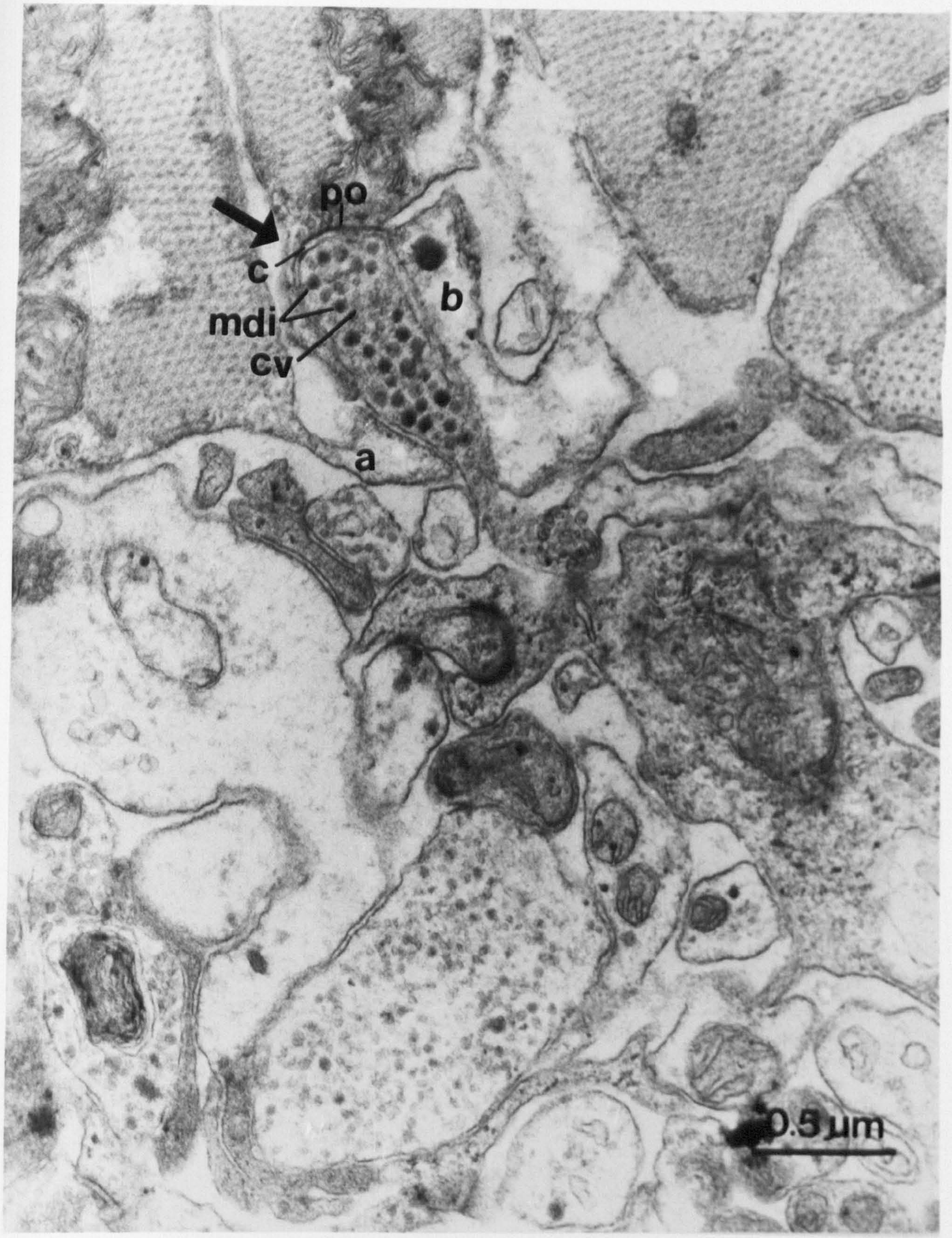

pp
c
mdi
cv
b
a
0.5 µm

Plate 8.12 Transmission electron micrograph of cercarial ocellus.

Longitudinal section through entire ocellus.

The pigment cup cell (pc) surrounding a rhabdomere (rh) extending from the retinular cell body (rc). The retinular cell body contains mitochondria, microtubules and granular endoplasmic reticulum. The pigment cup cell cytoplasm contains numerous pigment granules (pg), mitochondria (m) and golgi body (gb)

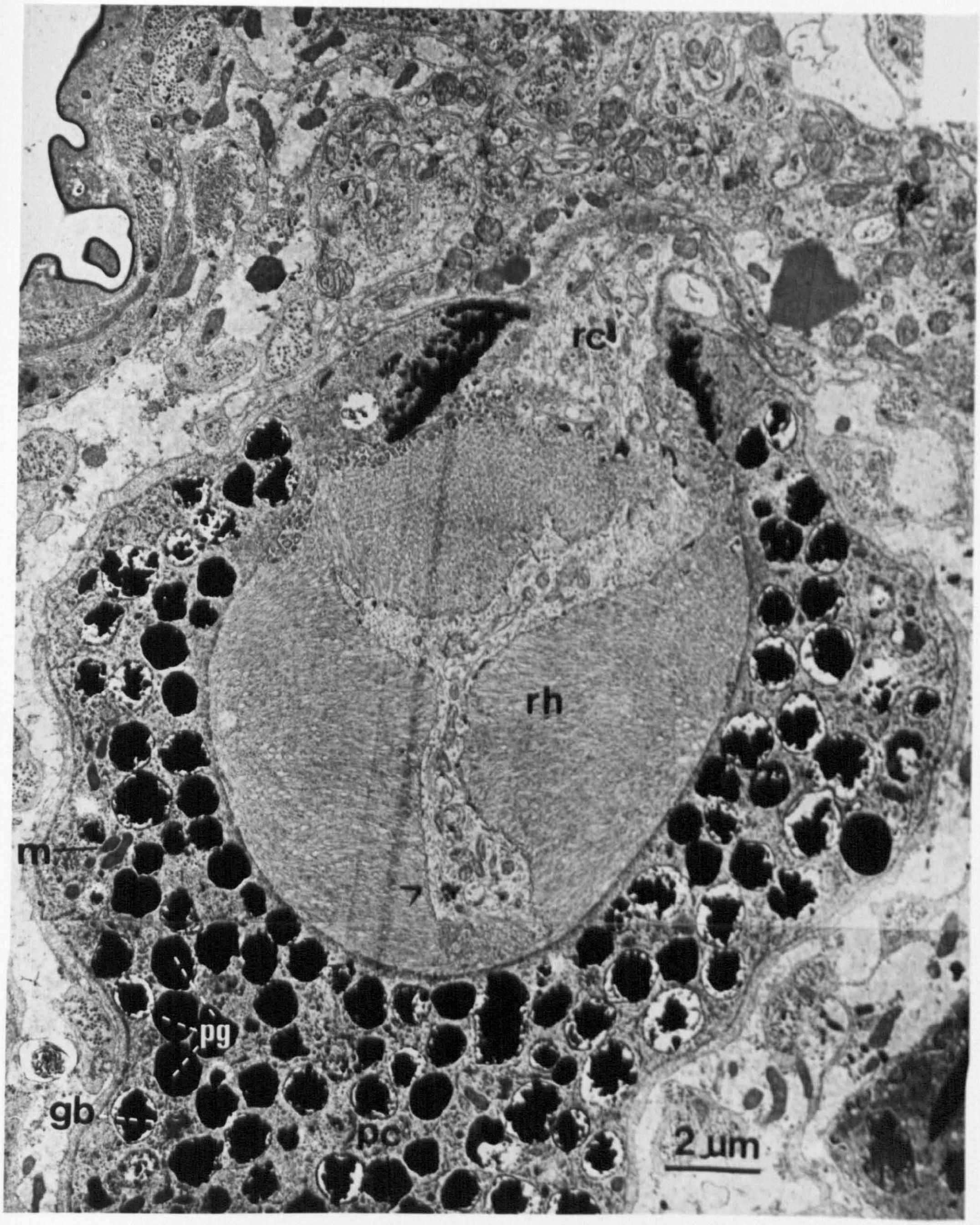

rcl
rh
m
pg
gb
pc
2 µm

Plate 8.13 Transmission electron micrograph of cercarial ocellus showing the retinular cell nucleus (rn) and the pigment cup cell nucleus (pn)

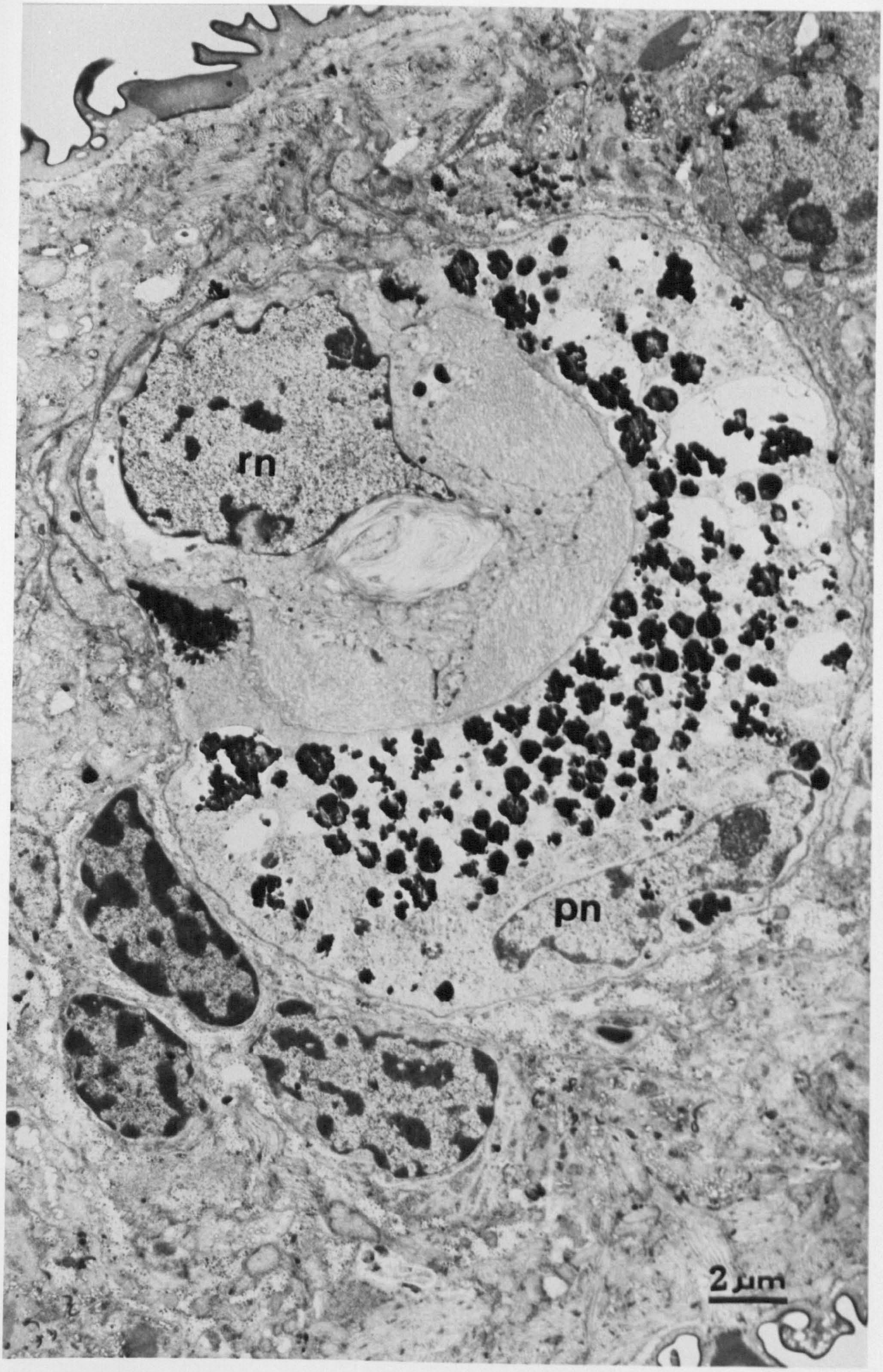

rn
pn
2 µm

Plate 8.14 Transmission electron micrograph of cercarial ocellus

(A) Cross section through rhabdomere showing internal structure of microvilli

(B) Electron dense striated material (dm) near the opening of the pigment cup and close to the presumed desmosome (d) between a rhabdomeric cell and the pigment cup cell

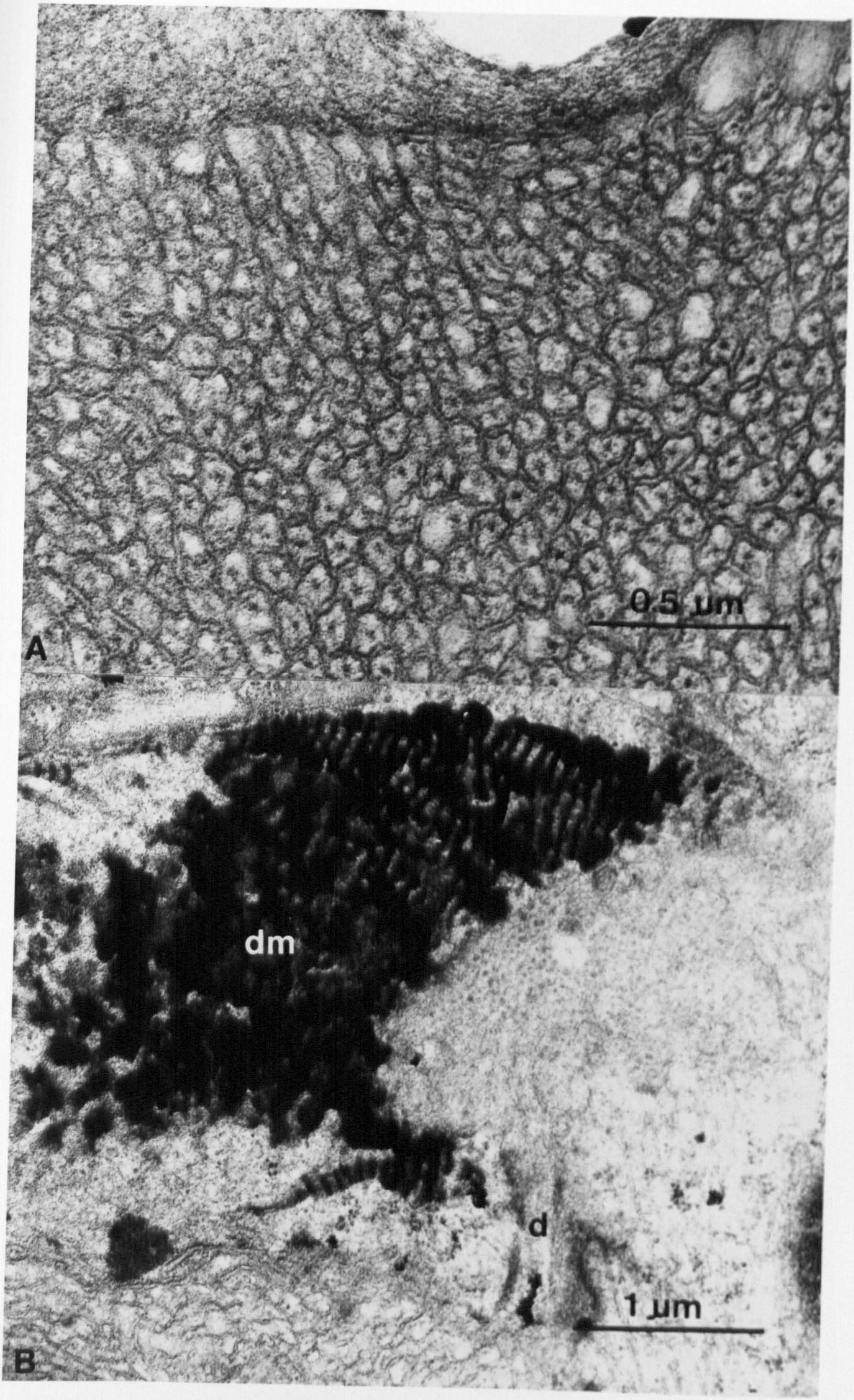
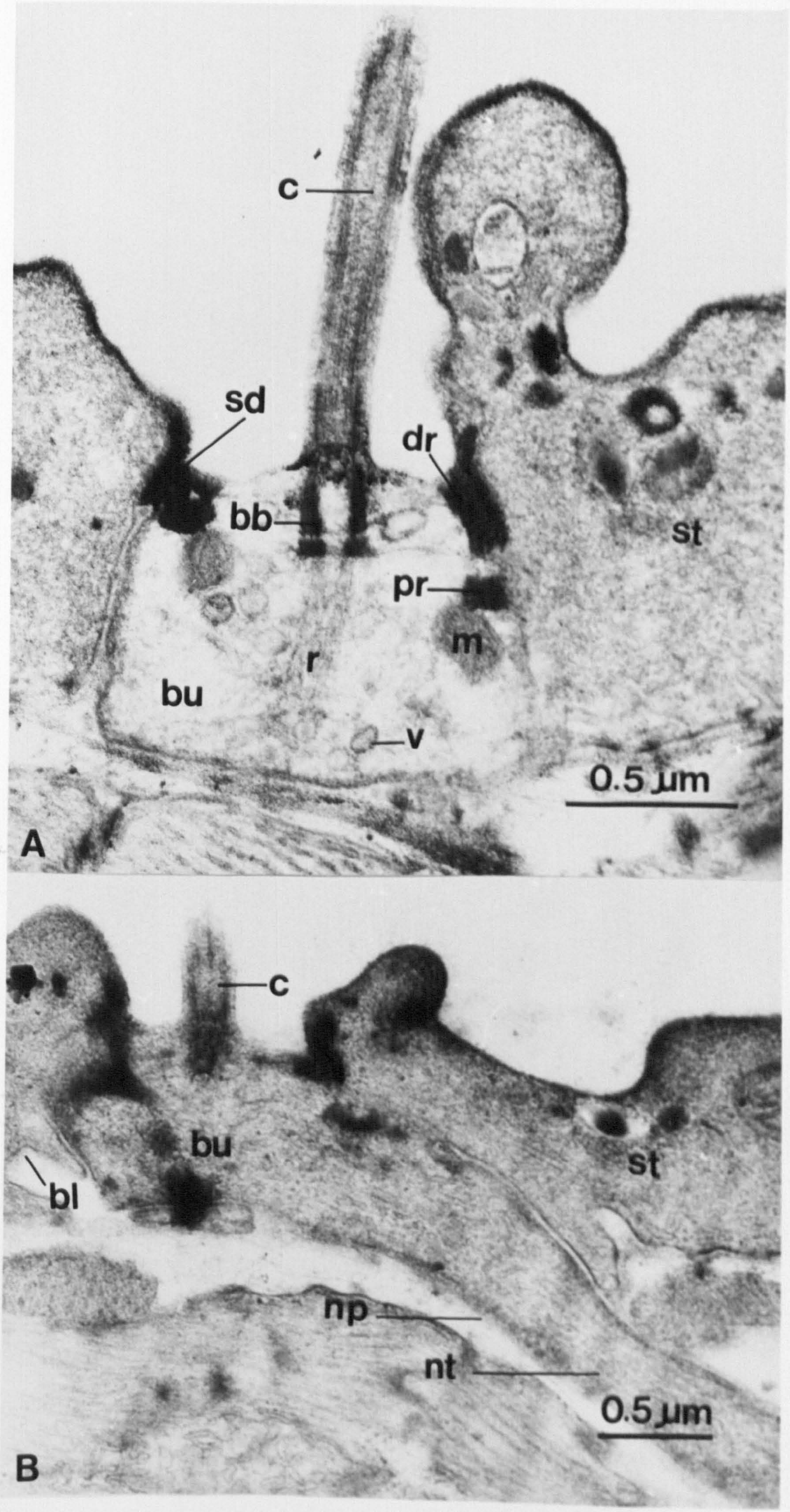

0.5 µm
A
dm
d
1 µm
B

Plate 8.15 Transmission electron micrographs of caudal ciliated surface receptors

(A) Furcal uniciliate receptor
bb: basal body of single sensory cilium
bu: nerve bulb
c: sensory cilium
dr: distal ring of dense material in nerve bulb
m: mitochondrion
pr: proximal ring of dense material in nerve bulb
r: striated rootlet of cilium
sd: septate desmosome
st: surface tegument of furca
v: electron lucid vesicle

(B) Tail stem uniciliate receptor showing portion of sensory nerve processes
bl: basal lamina
bu: nerve bulb
c: sensory cilium
np: sensory nerve process
nt: neurotubules within the nerve process
st: surface tegument of the tail stem


c'
sd
bb
dr
st
pr
m
r
bu
v
0.5 µm
A
c
bu
st
bl
np
nt
0.5 µm
B

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